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MANAGEMENT OF NEUROGENIC DETRUSOR OVERACTIVITY CAUSED BY SPINAL CORD INJURY

THE CASE OF BOTULINUM TOXIN AND RESINIFERATOXIN

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TESE DE DOUTORAMENTO EM NEUROCIÊNCIAS

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À Professora Doutora Célia Cruz

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Aos meus pais

À minha irmã

Ao Hugo

Prólogo

"*You have within you the strength, the patience and the passion to reach for the stars to change the world*"
Harriet Tubman

Sinto-me forçada a concordar com quem de perto me acompanha na vida, a palavra "porquê" é, muito provavelmente, uma das que melhor me definem. Memórias da minha infância espelham a imagem de uma criança curiosa que com notável empenho partia à descoberta do significado das mais variadas coisas. Foi o domínio das ciências que sempre me cativou, fascinava-me o estudo do organismo humano e as reações químicas desenvolvidas nos laboratórios da Escola Secundária Abel Salazar. Desse modo, foi-me bastante simples, e até intuitivo, traçar um plano para o futuro, traduzido na frase "*um dia vou ser cientista*".

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Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

Coelho A, Oliveira R, Cruz F, Cruz CD (2016) Impairment of sensory afferents by intrathecal administration of botulinum toxin A improves neurogenic detrusor overactivity in chronic spinal cord injured rats. Experimental Neurology, 285, 159-166.

Oliveira R, Coelho A, Charrua A, Avelino A, Cruz F (2017) Expression of cleaved SNAP-25 after bladder wall injection of onabotulinumtoxina or abobotulinumtoxina: A comparative study in the mice. Neurourology and Urodynamics, 36(1), 86-90.

Oliveira R, Chambel S, Cavaleiro H, Silva R, Cruz F, Cruz CD (2019) Effects of Early Bladder Administration of Botulinum Toxin A On Neurogenic Detrusor Overactivity Following Spinal Cord Injury. (Unpublished manuscript).

Oliveira R, Coelho A, Franquinho F, Sousa MM, Cruz F, Cruz CD (2019) Effects of early intravesical administration of resiniferatoxin to spinal cord-injured rats in neurogenic detrusor overactivity. Neurourology and urodynamics, 38(6), 1540-1550.

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List of abbreviations

Abobot/A – abobotulinumtoxinA

BDNF – brain derived neurotrophic factor

BPH – benign prostatic hyperplasia

BPS – bladder pain syndrome

BoNT/A – botulinum toxin A

CGRP – calcitonin gene-related peptide

DRG – dorsal root ganglia

DSD – detrusor sphincter dyssynergia

EC50 – half maximal effective concentration

EUS – external urethral sphincter

HC – heavy chain

Incobot/A – incobotulinumtoxinA

LC – light chain

LD50 – median lethal dose

LUT – lower urinary tract

NDO – neurogenic detrusor overactivity

NGF – nerve growth factor

NMJ – neuromuscular junction

NO – nitric oxide

OAB – overactive bladder

Onabot/A – onabotulinumtoxinA

PACAP - Pituitary adenylate cyclase-activating polypeptide

PAG – poliaqueductal gray

PMC – pontine micturition centre

RTX - resiniferatoxin

SCI – spinal cord injury

SNAP-25 - synaptosomal nerve-associated protein 25

SP – substance P

SV2 - synaptic vesicle glycoprotein 2

TrkA - tropomyosin receptor kinase A

TrkB - tropomyosin receptor kinase B

TRPV1 – transient receptor potential vanilloid 1

UI – urinary incontinence

VAMP - vesicle associated membrane protein

Abstract

Urinary dysfunction is a very common and one of the most debilitating consequences of spinal cord injury (SCI), being in the top list of treatment priorities. The direct damage to central nerves implicated in the control of lower urinary tract (LUT) lead to bladder areflexia and voiding deficiency that is subsequently overcome by neurogenic detrusor overactivity (NDO). This condition affects nearly 90% of SCI patients and is characterized by periods of extremely high intravesical pressures, which compromise kidneys' integrity, and frequent episodes of urinary incontinence that strongly affect their quality of life. Thus, management of NDO is of foremost importance and still a clinical challenge.

Botulinum toxin A (BoNT/A) is a powerful tool in the management of hyperactive muscles and is currently the gold standard treatment for NDO, for patients who are refractory to antimuscarinics, the first line of therapy. Numerous studies support the efficacy of bladder wall injections of BoNT/A in reducing bladder pressure and episodes of urinary incontinence. BoNT/A therapeutic effects last for several months and produce tremendous positive impact in patients' quality of life. However, severe urinary retention is still commonly pointed as an adversity of the treatment that needs to be overcome. In **Publications I** and **II** included in this dissertation we addressed that issue and conducted research aiming to improve BoNT/A-based treatment strategies.

In **Publication I** we defined an accurate conversion ratio for bladder-wall administration of the two BoNT/A formulations mostly used in clinics, onabotulinumtoxin/A (Onabot/A) and abobotulinumtoxin/A (Abobot/A). Onabot/A has been particularly well studied and it is the unique toxin product licensed for the treatment of NDO. Thus, doses of Abobot/A used in clinical urology are inaccurately established based on unspecific conversion ratios. With a fine analysis of toxin catalytic activity on SNAP-25, the specific target of the toxin, in bladders of mice

receiving each one of the formulations, we established a proper comparison and concluded that 1 unit (U) of Onabot/A is equivalent to 1.6 U of Abobot/A.

In **Publication II** we tested if intrathecal administration of BoNT/A, which restricts the toxin effect to the central processes of bladder sensory afferents, can be used to improved urinary dysfunction following SCI. This approach resulted in impairment of bladder afferents, implicated in NDO emergence and maintenance, and significant improvements in urodynamics, without affecting parasympathetic bladder innervation. These results support that restricting the action of the toxin to the sensory innervation of the bladder is still effective in improving bladder function without causing urinary retention.

As prevention is always better than cure, we placed additional efforts in studying possibilities to prevent NDO with neurotoxins-based treatments initiating early after SCI. We tested if bladder injections of BoNT/A during spinal shock were capable of modulate the emergence of bladder dysfunction (unpublished observations). We failed to find significant urodynamic improvements after early treatment with BoNT/A, in line to absence of toxin-mediated effects in bladder innervation and central neuronal projections. This most probably reflects incapacity of affected silent neurons to internalize the toxin.

In **Publication III**, we tested if intravesical resiniferatoxin (RTX) is effective in NDO prevention. RTX is a strong desensitizing agent of the TRPV1 channel, upregulated in the bladder of NDO patients and contributing to its pathophysiology. We found that the early treatment with RTX lead to significant urodynamic improvements, with decreased frequency of bladder contractions and marked reduction of intravesical pressures. These beneficial treatment outcomes resulted from bladder TRPV1 desensitization and consequent modulation of nerve sprouting. Effects were restricted to the bladder and support the use of RTX at early stages of disease progression to prevent degradation of urinary function.

Resumo

A disfunção urinária é uma das mais comuns e debilitantes consequências das lesões medulares, ocupando um lugar de topo na lista das prioridades de tratamento. O dano direto nas vias neuronais do sistema nervoso central implicadas no controlo do trato urinário inferior resulta numa situação de areflexia da bexiga, e consequente perda da capacidade urinária, que, subsequentemente, dá lugar à condição de hiperactividade neuronénica do detrusor (NDO). Esta patologia afeta cerca de 90% dos indivíduos que vivem com uma lesão medular e caracteriza-se por períodos de pressões intravesicais extremamente elevadas, que comprometem a integridade renal, e por episódios frequentes de incontinência urinária que afetam severamente a qualidade de vida dos doentes. Assim, o tratamento da NDO é de extrema importância e continua a ser um desafio para a prática clínica.

A toxina botulínica do tipo A (BoNT/A) é uma ferramenta poderosa no tratamento da hiperatividade muscular e é atualmente o tratamento de excelência da NDO em doentes que não respondem à terapia de primeira linha com antimuscarínicos. Até à data, são múltiplos os estudos que demonstram a eficácia de injeções de BoNT/A na parede da bexiga na redução da pressão intravesical e no número de episódios de incontinência urinária. Os efeitos terapêuticos da toxina são extraordinariamente duradouros e têm um impacto muito significativo na melhoria da qualidade de vida dos doentes. Contudo, a retenção urinária que advém da administração da BoNT/A permanece um problema que é necessário ultrapassar. Com foco nessas questões, os trabalhos que constam das **Publicações I** e **II** incluídas na presente dissertação foram desenvolvidos com o objetivo de melhorar as estratégias terapêuticas que se baseiam na utilização da BoNT/A.

Na **Publicação I** definiu-se um fator de conversão de doses concreto para a injeções na bexiga das duas formulações de BoNT/A maioritariamente utilizadas na clinica, onabotulinumtoxin/A (Onabot/A) e abobotulinumtoxin/A (Abobot/A). A Onabot/A tem vindo a

ser particularmente estudada e é a única formulação que se encontra aprovada por autoridades competentes no tratamento da NDO. Consequentemente, as doses de Abobot/A utilizadas na prática clínica baseiam-se em fatores de conversão aproximados e inespecíficos. Com base na fina análise da atividade catalítica da toxina no seu alvo específico, a proteína SNAP-25, em bexigas de murganhos tratados com cada uma das formulações estabeleceu-se um fator de comparação concreto e concluiu-se que 1 unidade de Onabot/A corresponde a 1.6 unidades de Abobot/A.

Na **Publicação II** estudou-se se a restrição do efeito da BoNT/A aos ramos centrais dos aferentes da bexiga, através de administração por via intratecal, resultava na melhoria da disfunção urinária que decorre de lesão medular. Esta abordagem levou à modulação dos aferentes vesicais, que estão implicados no aparecimento e manutenção da NDO, e resultou em melhorias significativas do padrão urodinâmico, sem afetar a inervação parassimpática da bexiga. Estes resultados sustentam a ideia de que a restrição da atividade da toxina à inervação sensitiva da bexiga é eficaz na melhoria da função vesical sem risco de ocorrência de retenção urinária associada.

Porque a prevenção é sempre melhor do que a cura, colocaram-se esforços adicionais no estudo de possibilidades de prevenir o aparecimento de NDO com recurso a tratamentos com neurotoxinas numa fase precoce pós-lesão. Inicialmente testou-se se a administração de BoNT/A na bexiga durante a fase de choque espinhal levava à modulação do aparecimento da disfunção urinária (**observações não publicadas**). O tratamento precoce com a toxina não resultou em alterações urodinâmicas significativas, o que se relacionou com a ausência de qualquer efeito na inervação da bexiga e nas respetivas projeções centrais desses neurónios. Estas observações refletem, muito provavelmente, a incapacidade de neurónios silenciados, afetados pela lesão, internalizarem a toxina.

Na **Publicação III** testou-se se a administração intravesical da resiniferatoxina (RTX) era eficaz na prevenção da NDO. A RTX é um potente agente dessensibilizador do recetor TRPV1,

que se sabe ter expressão aumentada na bexiga de doentes com NDO, contribuindo para a sua patofisiologia. Concluiu-se que o tratamento precoce com RTX resulta na melhoria significativa da função urodinâmica, levando ao decréscimo do número de contrações da bexiga e à redução significativa das pressões intravesicais. Estes efeitos resultam da dessensibilização do recetor TRPV1 e da consequente modulação do crescimento neuronal, exclusivamente ao nível da bexiga, e sustentam o uso da RTX em fases iniciais da progressão da doença, no sentido de prevenir a deterioração da função vesical.

State of the art

The complexity of the nervous system reflects its ability to control all and each part of the body. Control of lower urinary tract (LUT) function depends on complex neuronal pathways, easily jeopardized by spinal cord injury (SCI). Because it interrupts neuronal network regulating LUT function, SCI frequently leads to urinary dysfunction, a major concern for SCI patients. Treatment is often unsatisfactory and a major goal of the present work was to pave the way for improving available treatments and also investigate the possibility to harness NDO emergence.

1. Neuronal pathways innervating the lower urinary tract

The LUT comprises the urinary bladder, a reservoir for urine storage, and an outlet structure, including bladder neck, urethra and striated muscles of the external urethral sphincter (EUS), which contract and relax to block or permit voiding [1]. In newborns, urine release is a reflexive process, exclusively dependent on a primitive lumbosacral neuronal pathway [2]. Voluntary control of micturition develops with the maturation of the central nervous system and depends on learned behaviour, acquired in early childhood [2]. In adults, voluntary switching between storage and voiding relies on the synchronised contraction and relaxation of bladder and outlet structures. This coordination is only achieved via activation of intricate neuronal pathways, involving peripheral and centrally located neurons [3, 4].

In Humans and experimental animals, the LUT is innervated by autonomic sympathetic and parasympathetic nerves, as well as somatic motor and sensory fibres [3, 4]. Sympathetic noradrenergic fibres originate in thoracolumbar segments of the spinal cord, run through the inferior mesenteric plexus and hypogastric nerve and induce bladder relaxation via activation of β3 receptors [5]. These receptors are expressed by bladder smooth muscle cells [6] and acetylcholine-containing nerve fibres [7]. Sympathetic fibres also run through paravertebral chain, enter pelvic nerves to reach the bladder neck and urethra. These fibres, exerting their effect via α1 receptors, are apposed to the smooth muscle fibres of the internal urethral sphincter [5]. Parasympathetic preganglionic fibres arise from neurons located in sacral spinal

segments and run through sacral roots and pelvic nerves, reaching post-ganglionic neurons, which are positioned intramurally in the Human bladder. Axons emerging from neurons located in these ganglia innervate the detrusor muscle. Both preganglionic and postganglionic neurones release acetylcholine, which activate nicotinic receptors in postganglionic neurons and muscarinic receptors in bladder smooth muscle cells [5, 8]. The urethra is also innervated by postganglionic nerves, which release nitric oxide (NO) to induce relaxation [3, 9]. Finally, somatic cholinergic motor nerves that originate in sacral spinal cord segments travel through pudendal nerves, contributing to urethral contraction through activation of EUS nicotinic receptors [1, 10].

While efferent fibres regulate the cycles of contraction/relaxation necessary for proper LUT function, afferent neurons sense bladder stretch. Their cell bodies are located in lumbosacral dorsal root ganglia (DRG) and they convey neuronal inputs from the LUT to second order neurons located in the spinal cord [1, 11]. This sensory input reaches the thoracolumbar spinal cord carried by afferents travelling in the hypogastric nerve and the lumbosacral segments of the spinal cord, via the pelvic nerve. Bladder sensory afferents are glutamatergic [12-14] and can be categorized into Aδ and C-fibres. Aδ afferents have small-sized but larger cell bodies than C-fibres and their processes are covered by a thin myelin sheath resulting in higher conduction velocity [15, 16]. These afferents respond to physiological bladder filling, such as passive bladder-wall distention and active detrusor contractions [17]. C-fibres have small sized cell bodies and their axons are unmyelinated. The majority of them are peptidergic, expressing substance P (SP), calcitonin-gene related peptide (CGRP) and pituitary adenylate cyclase activating polypeptide (PACAP). In addition, these peptidergic fibres also express numerous receptors, including transient receptor potential vanilloid 1 (TRPV1), tropomyosin receptor kinase A (TrkA) and B (TrkB), the high affinity receptors for nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), respectively, and purinergic receptors [18]. While in normal conditions C-fibres have minor contribution, they have an important and well-established role in the pathophysiology of multiple LUT syndromes, such as urinary dysfunction arising from SCI.

1.1. Neuronal control of urine storage and voiding

The LUT operates in a switch-like fashion, without intermediate stages between voiding and continence. In normal physiological conditions, during storage the bladder undergoes low-pressure filling until it retains approximately 300 to 400 mL of urine [19]. During bladder filling, intravesical pressures are not high enough to generate a strong sensory input. This low intensity sensory firing is conveyed to spinal interneurons and from there to the L-region of the pontine storage centre, located in the pons. During bladder filling, continence results from sympathetic stimulation of the smooth muscle of bladder neck and urethra, through the hypogastric nerve, and somatic activation of the EUS via pudendal nerves, which keeps the outlet contracted to avoid urine leakage [1] (***Figure 1a***).

When bladder reaches its maximal capacity, bladder afferents are activated and a high intensity sensory input is generated, which is conveyed to the spinal cord and supraspinal structures, including the cortex. Once a decision to void is made, the pontine micturition centre (PMC) is activated. Its descending projections inhibit sympathetic and somatic urethral innervation, inducing relaxation of the urethral sphincter. In turn, preganglionic parasympathetic neurons at sacral spinal cord are stimulated, generating parasympathetic outflow to induce detrusor contraction and urine release (***Figure 1b***). Of note, in healthy individuals, micturition is under voluntary control, which require activation of important supraspinal nuclei. Accordingly, the activation of the cingulate gyrus and insula has been reported following perception of bladder volume [20]. Those areas are known to indirectly modulate PMC processing via the periaqueductal gray matter (PAG) [1]. Thus, when voiding is appropriate, the prefrontal cortex interrupts the tonic suppression of PAG input to the PMC. This leads to interruption of the sympathetic input to the detrusor and parasympathetic drive to the urethral sphincter, resulting in bladder contraction and urethral relaxation, necessary for urine expulsion.

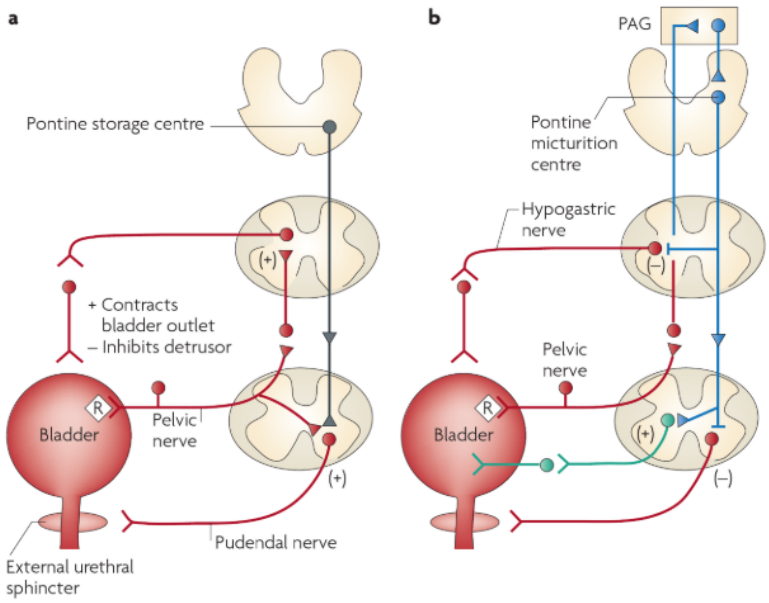

Figure 1 – Neuronal pathways implicated in urine storage (**a**) and voiding (**b**). Sympathetic efferent and sensory afferent innervation from the spinal cord to bladder and urethra is depicted in **red**. Spinobulbospinal reflex pathways are represented in **blue**. Parasympathetic outflow to the bladder is depicted in **green**. Fowler et al., 2008 [1].

2. Spinal cord injury and urinary dysfunction

2.1. Prevalence, causes and consequences of spinal cord injuries

SCI is a devastating event with a tremendous impact in patients' life. It is currently estimated that more than 2 million people worldwide live with SCI and World Health Organization projects 40 to 80 new cases per millions of people every year [21, 22]. Non-traumatic events such as spinal stenosis, tumours, vascular ischemia and congenital diseases may lead to SCI. However, traumatic injuries related to motor vehicle accidents, falls, sports and violence are the most common causes [23, 24]. SCI represents an enormous burden to individuals and overall economy [25]. Although improved medical treatment has greatly increased post-injury survival, patients present high rates of incapacity and need costly daily care support [22, 26].

The majority of spinal lesions are incomplete and occur at cervical levels of the cord [22], causing a disruption of neuronal pathways that control multiple body systems. For that reason, SCI is typically associated with substantial impairment or complete loss of motor, sensory and autonomic functions. Among all consequences, LUT dysfunction is one of the most prevalent, affecting nearly 90% of patients and severely compromising their quality of life. Accordingly, it is often referred by patients as one of the most incapacitating aspects in post-injury everyday life [27-29]. Management of urinary dysfunction is fundamental in early and late stages post-injury, as it might result in serious complications such as life-threatening kidney failure and frequent urinary infections. In addition, LUT dysfunction also has a tremendous impact on social life, either because patients fear the occurrence of episodes of urinary incontinence or due to the permanent need of being close to a toilet room [30].

While during spinal shock disruption of LUT parasympathetic outflow leads to bladder hypocontractility, disease progression often results in bladder hyperactivity [3]. It is estimated that nearly 95% of SCI patients develop neurogenic detrusor overactivity (NDO), a pathological condition characterized by strong and frequent bladder contractions detected in urodynamic evaluations [31-34], accompanied by frequent episodes of urinary incontinence (UI) that deeply influence their everyday life . Moreover, due to the loss of coordination between bladder and EUS, known as detrusor sphincter dyssynergia (DSD), patients experience periods of very high and extremely dangerous intravesical pressures that produces significant damage to the upper urinary tract. Therefore, treatment of SCI-induced urinary dysfunction is of foremost importance, topping the list of priorities for patients and caregivers and remaining a major challenge in rehabilitation units.

2.2. Mechanisms underlying neurogenic detrusor overactivity and detrusor-sphincter dyssynergia

Spinal injury above lumbosacral levels acutely disrupts voluntary voiding and supraspinal LUT control. Loss of descending inputs from supraspinal regulatory centres triggers bladder areflexia and continuous urine storage [35]. With time, the majority of patients regain automatic voiding capacity, which is linked to NDO and DSD emergence [36] (***Figure 2***).

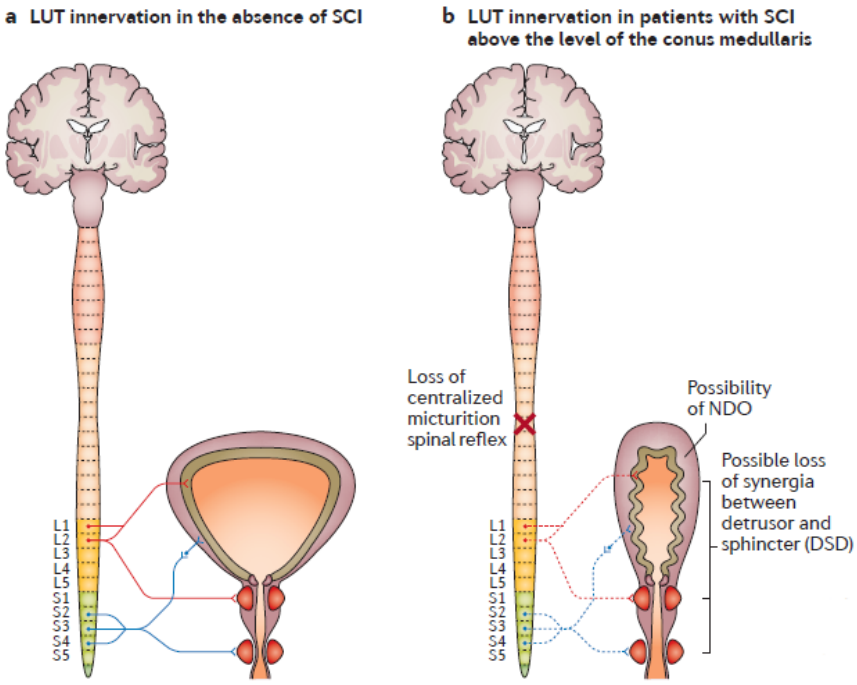

Figure 2 – Schematic representation of lower urinary tract innervation in a normal situation (**a**) and after a spinal cord injury above lumbosacral segments (**b**). Adapted from Wyndaele et al., 2016 [36].

The reasons underlying the development of such conditions have been extensively studied and loss of bulbospinal inhibitory inputs, synaptic rearrangement, axonal growth, changes in neurotransmitter pharmacology and modifications in afferent firing patterns are thought to concur in NDO and DSD development and maintenance [37-39]. These processes are dependent on the effects of neurotrophins, including NGF and BDNF, on the neuronal circuits governing LUT function [40-42].

Because of such neuroplastic events, a new micturition reflex is established, exclusively mediated by type C unmyelinated bladder afferents [43-46] (***Figure 3***). The involvement and importance of C fibres is further strengthened by clinical trials demonstrating that desensitization of TRPV1 expressing fibres markedly reduced NDO [47-50].

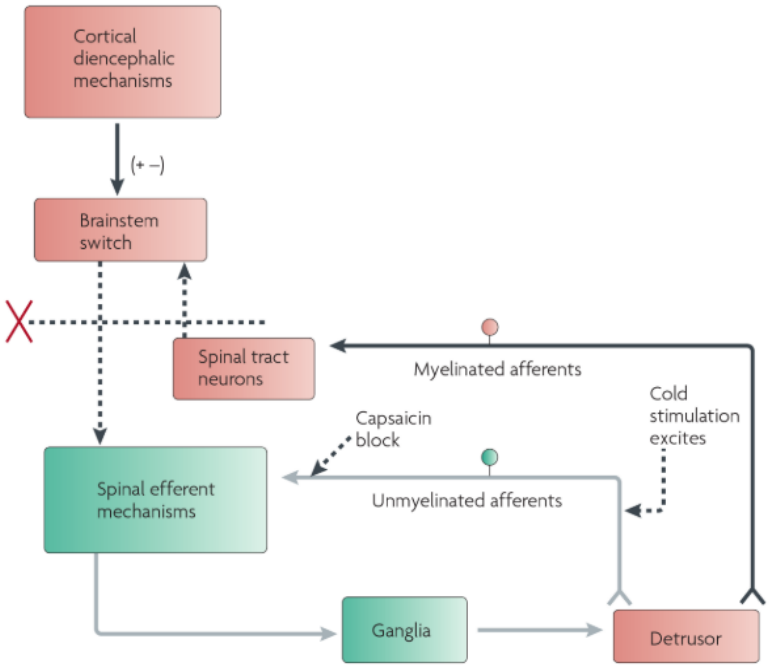

Figure 3 - Schematic representation of the afferent pathways conveying neuronal inputs from the bladder to the supraspinal regulatory centres (**Red boxes** and **black arrows**) and the micturition reflex circuitry that emerges after spinal cord injury (**Green boxes** and **grey arrows**). Fowler et al., 2008 [1].

2.3. Management of bladder dysfunction after spinal cord injury

With marked improvements in survival of traumatic SCI patients [51], dealing with urinary dysfunction became an important priority. During spinal shock, the main concern is to prevent the rise of intravesical pressures and protect the upper urinary tract [36, 52]. Once NDO and DSD are installed, treatment aims to prevent urine leakage [34, 36]. For that, several approaches are available to reduce detrusor contractions and improve urine storage. These are

combined with intermittent catheterization, performed by the caregiver or the patient, for timely urine removal. However, available treatments are not exempt of bothersome secondary effects, including urinary retention and urinary tract infections [30].

NDO management is typically initiated with antimuscarinics, including oxybutynin, fesoterodine and darifenacin, aiming to reduce bladder hypercontractility [30, 36]. These drug block M2 and M3 muscarinic receptors, present in the detrusor muscle, and prevent acetylcholine-mediated detrusor contractility. However, anticholinergic medication is often associated with severe adverse effects, such as cognitive impairment, constipation, dry mouth, blurred vision and tachycardia, leading patients to discontinue treatments. Desmopressin, a vasopressin analogue, might also be useful as a therapeutic option due to its effectiveness in reducing urine production [36, 53]. More recently, agonists of β3 adrenoreceptors were licensed for the treatment of overactive bladder [54, 55]. While some investigators demonstrated improvement of urodynamic parameters in SCI patients and animals treated with a combination of antimuscarinic drugs with β3 agonists [56, 57], other studies failed to show significant benefits [58]. Sacral and tibial neuromodulation as well as electrical stimulation of the pudendal and dorsal genital nerves have also emerged as valuable therapeutic options for neurogenic bladder, with long-lasting effects [30, 59], but the underlying molecular mechanisms of neuromodulation are still poorly understood.

2.3.1. Neurotoxins in the treatment of bladder dysfunction after spinal cord injury

a) Botulinum toxin A (BoNT/A)

Bladder wall injections of BoNT/A is the current gold standard therapy for NDO patients that are refractory to first line treatments [30, 52]. By effectively blocking vesicle-mediated neurotransmission, this toxin leads to flaccid paralysis of bladder smooth muscle [60, 61]. The success of this treatment can be ascribed to its long-lasting effects, safe administration technique and lack of considerable adverse effects [61].

The mechanism of action of BoNT/A was firstly described for the neuromuscular junction (NMJ) [62, 63]. Composed by two peptide chains, a heavy and a light chain (HC and LC, respectively), this toxin is internalized by active axonal terminals. The HC binds to ganglioside receptors at the surface of presynaptic neurons, allowing docking of the neurotoxin to the presynaptic terminal. During neurotransmission, the synaptic vesicle glycoprotein 2A (SV2) is exposed and the toxin binds specifically to this protein [64]. As the synaptic vesicle is recycled, BoNT/A is internalized and the LC is released from the HC, acquiring proteolytic activity. The LC cleaves proteins necessary for docking and fusion of neurotransmitter-containing vesicles, including vesicle-associated membrane protein (VAMP) or synaptobrevin, syntaxin and synaptosomal nerve-associated protein of 25 kDa (SNAP-25) (***Figure 4***). In skeletal muscle, the blockade of neurotransmission of cholinergic fibres results in paralysis. Pioneer studies dating back to the 1980's showed that injections of the neurotoxin into hyperactive muscles induced site-limited temporary relaxation [65-67]. The restricted activity to the injection site, the low frequency of adverse effects and the long-duration in neuromuscular blockade support its therapeutic use in multiple spasmodic, painful and hypersecretory syndromes [68-70].

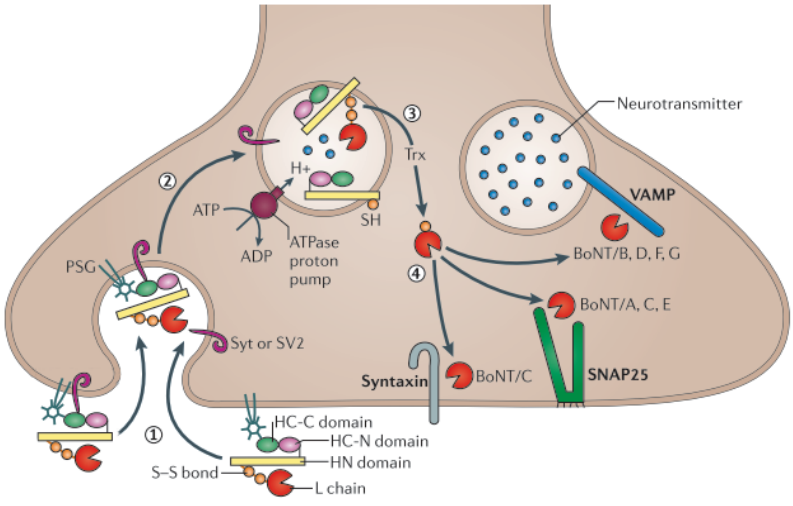

Figure 4 - Schematic representation of botulinum toxin internalization (**1**) in a presynaptic neuron through vesicle recycling (**2**) and subsequent events of neuronal intoxication, such as protoxin cleavage and light chain translocation to the cytosol (**3**) culminating in the cleavage of SNARE proteins (**4**). Adapted from Rossetto et al., 2014 [71].

BoNT/A is currently available in three distinct formulations, OnabotulinumtoxinA (Onabot/A), AbobotulinumtoxinA (Abobot/A) and IncobotulinumtoxinA (Incobot/A), commercialized with the trade names of Botox© (Allergan, Inc., Irvine, CA, USA), Dysport© (Ipsen Biopharm Ltd., Wrexham, UK) and Xeomin© (Merz PharmaceuticalsGmbH, Frankfurt, Germany), respectively. All formulations share the same active principle, consisting of the 150 kDa neurotoxin, but differ in their content on accessory proteins [70]. The efficacy of each formulation is thought to depend on the respective diffusion across the treated tissue and unitary doses are not interchangeable, making it difficult to establish a conversion ratio [72]. Therefore, accurate therapeutic dosages of each formulation should be carefully evaluated in the context of each application [70].

Botulinum toxin A in Neuro-urology

The initial works supporting the use of BoNT/A in SCI-related LUT dysfunction date from more than 20 years ago [73-75]. The pioneer studies by Schurch and co-workers demonstrated that injections of BoNT/A in the EUS alleviated DSD in chronic SCI patients, with improvements in post void residual volumes that were still present 3 to 9 months post-injections [74, 75]. The same research team also demonstrated significant improvements in urodynamics as well as decrease in urinary incontinence episodes after BoNT/A bladder-wall injections [76, 77]. Large, double-bind placebo-controlled studies were performed later and found a significant reduction in episodes of urinary incontinence and clear improvements in urodynamic parameters, such as detrusor compliance, bladder capacity and maximum voiding pressures [78-80]. Improvement of LUT function was long-lasting and markedly improved patients' quality of life. At the present, only the formulation Onabot/A has been approved by competent authorities for the treatment of NDO [72, 81].

In the bladder of humans and experimental animals, the targets of BoNT/A include parasympathetic cholinergic, sympathetic adrenergic and sensory peptidergic fibres [82-84].

This explains not only its impact on reducing bladder contractions but also its antinociceptive effects [85-87]. In fact, improvement of bladder function and pain reflect a decrease in CGRP release from bladder afferents [88], as well as a reduction in the expression of $P2X_3$ and TRPV1 receptors in bladder fibres [89] and the blockade of neurotransmitters release from sensory fibres [60, 90] (***Figure 5***).

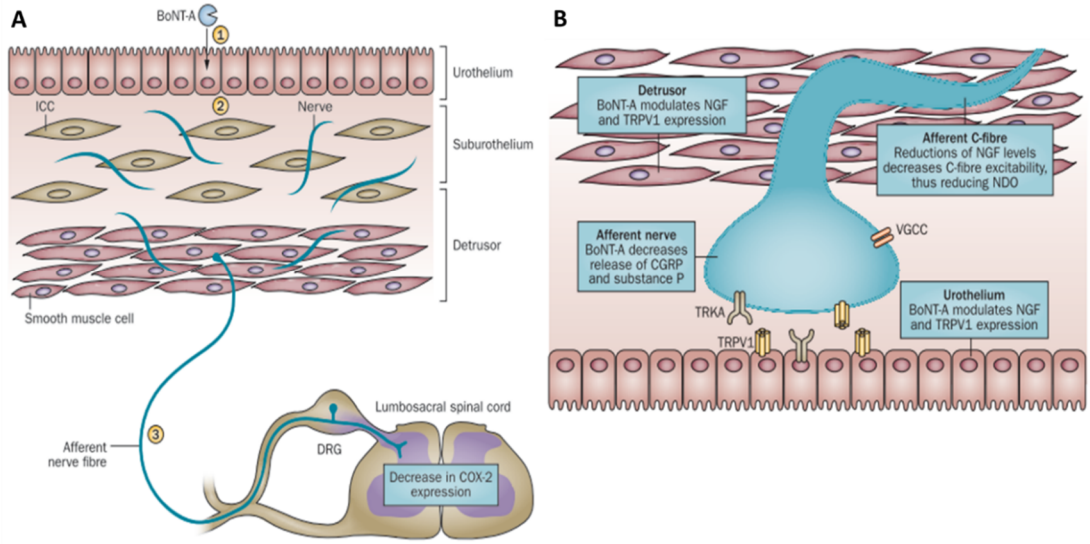

Figure 5: Sensory effects of BoNT/A. (**A**) Accumulation of the toxin in the urothelium (**1**) and inhibition of ATP release in the bladder (**2**) followed by internalization and retrograde axonal transport through bladder sensory afferents (**3**). (**B**) Bladder administration of BoNT/A diminishes the release of neuropeptides by afferent terminals and modulates NGF and TRPV1 expression in the bladder and lumbosacral spinal cord. Adapted from Jiang el al., 2015 [85].

Although debatable, some researchers suggest that BoNT/A activity is not restricted to the injection site and hypothesize that the neurotoxin is transported and acts at distant locations, carried via blood flow or axonal transport [91]. Accordingly, the presence of cleaved SNAP-25, an established marker of BoNT/A activity [82], was detected in retina, fascial nucleus, superior colliculus and bladder-wall after being administered in the optic tectum, whisker muscles, eye and lumbosacral intrathecal space, respectively, supporting that BoNT/A can

undergo retrograde and anterograde transport [92-95]. Therefore, some authors suggest that the pronounced efficacy of BoNT/A administration relies not only on its peripheral but also on central effects [96].

b) Resiniferatoxin

Resiniferatoxin (RTX) belongs to a family of compounds known as vanilloids. This family of molecules includes capsaicin, the pungent extract obtained from capsicum peppers. Like capsaicin, RTX is an agonist of the TRPV1 receptor [97]. This ion channel is a polimodal receptor [98, 99] present in bladder afferents and urothelial cells [100-102]. TRPV1 activation by intravesical administration of vanilloids, including RTX and capsaicin, results in long-lasting desensitization of bladder afferents [101, 103] (***Figure 6***).

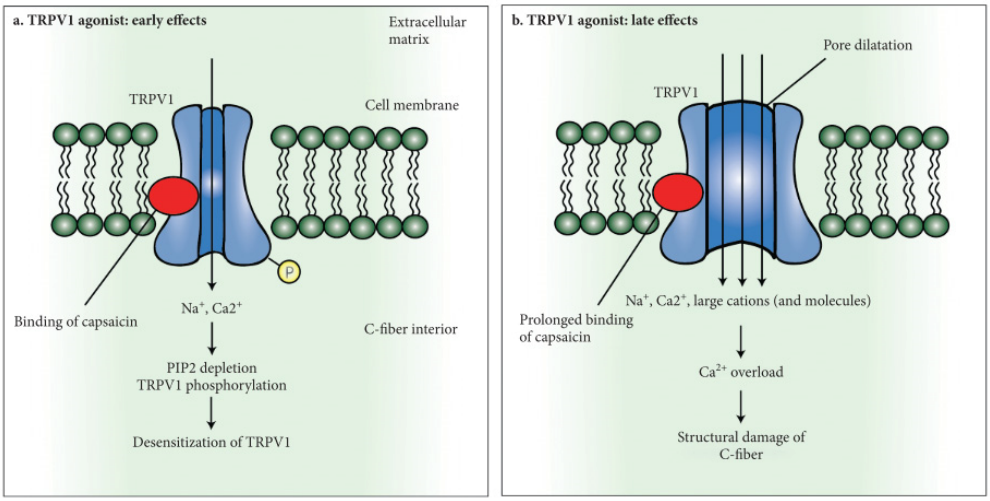

Figure 6: Prior desensitization of TRPV1 expressing fibres provoked by a short contact with channel agonists (**a**) and later effects producing profound changes in such fibres resulting from the prolonged administration of TRPV1 agonists (**b**). Adapted from Deruyver et al, 2015 [104].

In comparison to capsaicin, RTX-induced TRPV1 desensitization is more pronounced and effective, but surprisingly, treatment is less painful. Indeed, capsaicin has also been used to desensitize bladder sensory afferents [48, 105], but the pungency of its solutions has hampered

its clinical application, with clinicians preferring RTX. Because it has a higher affinity for TRPV1, RTX can be used in nanomolar concentrations without causing any burning sensation [106].

Resiniferatoxin in Neuro-urology

Capsaicin was initially intravesically applied in patients with bladder pain, detrusor hyperreflexia and neurogenic bladder [47, 48, 106-108] but its use was discontinued due to its highly irritant properties. In subsequent studies, RTX was preferred as it is more potent and less pungent than capsaicin [105, 109]. In patients presenting bladder hyperreflexia due to CNS pathology or insult, intravesical RTX, used in concentrations of 50 to 100 nmol/L, resulted in marked improvements in urinary symptoms [110]. Lazerri et al. also showed significant improvements in bladder capacity, frequency, nocturia and incontinence in patients diagnosed with bladder hyperactivity, which were still evident 4 weeks after treatment [111]. In both studies, treatment was not considered painful, with patients only referring minor itching and mild discomfort during RTX administration. In animals, instillation of 100nM of RTX in rats' bladders resulted in effective bladder afferent desensitization without significant increase in spinal c-Fos, a well-defined marker of noxious input [112]. Several other studies supported the clinical use of intravesical RTX, particularly in patients with NDO caused by spinal cord injury and multiple sclerosis, with improvement of bladder function and increased protection of the upper urinary tract [49, 50, 106, 113]. Similar observations were obtained from patients with idiopathic detrusor overactivity [114]. Still it was not possible to produce a stable and affordable formulation and with an upraise of BoNT/A as the treatment of choice for NDO, RTX is now seldom used [115].

Main goals

From the review above, it is clear that several therapeutic tools for NDO management have been developed over the years. Treatment is usually initiated with anticholinergic drugs, aiming to block detrusor contractions and making the bladder a flaccid reservoir to be periodically emptied by catheterization. For patients refractory to anticholinergics, BoNT/A has emerged as an excellent therapeutic choice. Of note, treatments are still not exempt of secondary effects and are not fully and/or permanently effective. Also a matter of concern is the fact that treatment is only initiated when urinary dysfunction is a fixed condition not likely to revert. Still, because prevention is better than cure, some research teams have initiated studies aiming to investigate if NDO emergence can be stopped. A recent study showed that administration of anticholinergics initiated immediately after SCI helped to hamper NDO development [116], in line with Sievert and co-workers who used neuromodulation to prevent NDO emergence [117].

This thesis had two main goals:

Goal 1: to improve treatment of chronic NDO.

Specifically, here the objectives were:

a) To compare the efficacy of two commercially available formulations of botulinum toxin A, Onabot/A and Abobot/A, and establish an accurate conversion ratio for clinical use, (***Publication I***)

b) To investigate the effects of intrathecal Onabot/A in NDO treatment (***Publication II***)

Goal 2: to investigate if NDO can be prevented.

Here, the points addressed were:

a) To assess if early bladder-wall injections of Onabot/A modulate NDO development (***unpublished observations****)*

b) To evaluate if early intravesical administration of RTX prevents NDO emergence (***Publication* III**)

Data obtained is presented and discussed according to a logical sequence, and not necessarily the date of publication.

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Publications and additional works

Publication I

Oliveira R, Coelho A, Charrua A, Avelino A, Cruz F (2017) Expression of cleaved SNAP-25 after bladder wall injection of onabotulinumtoxina or abobotulinumtoxina: A comparative study in the mice. Neurourology and Urodynamics, 36(1), 86-90.

Neurourology and Urodynamics 36:86–90 (2017)

Expression of Cleaved SNAP-25 After Bladder Wall Injection of OnabotulinumtoxinA or AbobotulinumtoxinA: A Comparative Study in the Mice

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Aims: To study the expression of cleaved synaptosomal associated protein of 25 kDa (cSNAP-25) in the bladder wall injected with onabotulinumtoxinA (onabotA) or abobotulinumtoxinA (abobotA) and compare the relative potency of these two brands. **Methods:** One injection of 0.5 U of onabotA or abobotA diluted in 2 μl of saline was carried out in the bladder dome of adult female mice, whose bladders were exposed by laparotomy. Three days later bladders were collected, divided in five segments (dome, upper, middle and lower body, and trigone) and each one was sectioned and immunoreacted against cSNAP-25, the end product of botulinum toxin type A (BoNT/A) activity. From each of the five segments one section was taken at random and the number of cSNAP-25 immunoreactive (IR) fibers was determined. **Results:** Each injection resulted in the cleavage of SNAP-25 in all bladder sections, including those of the more distant segment from the injection point. The average number of cSNAP-25 positive fibers was higher in the onabotA, 341 ± 301, than in the abobotA-treated mice, 208 ± 152 ($P = 0.003$). The number of cSNAP-25 IR fibers varied three to five-fold between animals of each experimental group. **Conclusions:** These findings confirm that, when injected in the bladder wall, in the same unit amount and same volume, onabotA is 1.6 times more potent to cleave SNAP-25 than abobotA. The conversion ratio suggested by these experiments is 1:1.6 between onabotA and abobotA. Each injection, although preformed in the same way, may induce substantially different amounts of cSNAP-25. *Neurourol. Urodynam. 36:86–90, 2017.* © 2015 Wiley Periodicals, Inc.

Key words: botulinum toxin type A; conversion ratio; cSNAP-25; relative potency; urinary bladder

INTRODUCTION

OnabotA is licensed for the treatment of neurogenic detrusor overactivity (NDO) and overactive bladder (OAB) in doses of 200 and 100 U, respectively.[1,2] The approved method for toxin administration is the bladder wall injection, distributed in 30 points for NDO and 20 points for OAB.[3]

This new form of drug administration raises two important questions. One relates to the replicability of the administration method, as bladder wall injection of BoNT/A clearly depends on the surgeon's technique. An incorrect administration may result in the deposition of the toxin outside the bladder or allow the backflow of the toxin solution through the injection hole. In both cases, the amount of toxin available for the impairment of bladder innervation will decrease, reducing the treatment efficacy. In fact, from a clinical point of view, this issue is extremely relevant, as in NDO regulatory trials 25% of patients did not show a clinically relevant reduction in the number of incontinence episodes following the first injection.[1,4] Also, in OAB regulatory trials 40% of the patients considered that the treatment did not improve their urologic condition.[5,6] These percentages are surprising if it is taken into consideration that the target for BoNT/A is the SNAP-25, a membrane protein abundantly expressed in all nerve fibers.[7] In addition, a recent subanalysis of NDO trials showed that nonresponders could have a good outcome to subsequent injections, suggesting that some variability in drug delivery may occur during bladder wall injection.[8]

Another problem related to bladder injections of BoNT/A is that, in addition to onabotA, abobotA and incobotulinumtoxinA (incobotA) are used off-label for the treatment of NDO and OAB. However, the doses are not interchangeable between these toxins making a conversion ratio impossible to establish.[9,10] The potency of each brand is established by distinct tests. Although onabotA potency is determined by the EC50 (amount of toxin required to provoke a response halfway between the baseline and the maximum response) of cSNAP-25 in differentiated neuroblastoma cells, abobotA potency is determined by the mouse LD50 (amount of toxin that kills 50% of the mice).[11–13] In the urological literature, the empiric conversion ratio of 1 U of onabotA to 3–5 U of abobotA is commonly suggested.[14,15] However, accurate comparative studies necessary to define the appropriate conversion ratio for bladder wall administration are lacking. Furthermore, onabotA and abobotA are protein complexes with hemagglutinin being an accessory protein in both cases. OnabotA vials contain 0.5 mg of human albumin and sodium chloride, whereas abobotA vials contain 0.125 mg of human albumin

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and lactose. IncobotA is a nude toxin.[10] The molecular weight of onabotA, abobotA, and incobotA is 900 kDa, approximately 500 kDa, and 150 kDa, respectively.[10,11,15] These differences may have consequences for the spread of the toxins across the bladder tissues, which, in turn, may influence the number of nerve fibers exposed to them at areas of the bladder more distant from the injection point.

This study has two aims, one primary and one secondary. The primary aim was to determine the relative potency of the two most commonly used BoNT/A brands, onabotA and abobotA, according to the amount of cSNAP-25 expression. The secondary aim was to investigate the variations of cSNAP-25 expression between individual animals.

MATERIALS AND METHODS

Animals

All experiments were performed in female C57BL/6J mice (Charles River, Barcelona, Spain) with an approximate weight of 20–30 g, maintained under a 12 hr light/12 hr dark schedule, with free access to food and water. All procedures were carried out according to the European Communities Council Directive (2010/63/EU) and all efforts were made to reduce the suffering and the number of animals used.

Reagents and Drugs

Surgeries were performed under isoflurane anesthesia (4% for induction and 1.5% for maintenance). A sodium pentobarbital overdose was used for terminal handling. BoNT/A formulations, onabotA (trade name: Botox®) and abobotA (trade name: Dysport®) were purchased from Allergan (Irvine, CA) and Ipsen Biopharm (Wrexham, UK), respectively. Rabbit anti-cSNAP-25, produced against the truncated C-terminal peptide of SNAP-25, was a kind gift from Ornella Rossetto and has been previously described.[16] The avidin–biotin complex (ABC) was purchased from Vector Labs (Peterborough, UK). Biotin-conjugated swine anti-rabbit was purchased from Dakopatt A/5, Dako, Lisbon.

Experimental Groups and Toxin Administration

Twenty-eight mice were anesthetized and their bladders were exposed by mid-line laparotomy. Bladders were manually emptied, to increase the thickness of the bladder wall, and one single injection was carried out in the bladder dome of each animal. The injection was performed with a NanoFil 10 μl syringe and a 35G beveled NanoFil needle (World Precision Instruments, Inc., Hertfordshire, UK). One experimental group (n = 12) received a single injection of 0.5 U of onabotA diluted in 2 μl of saline whereas the other group (n = 12) received a single injection of 0.5 U of abobotA diluted in 2 μl of saline. Control animals (n = 4) received a single injection of 2 μl of saline. All the injections were performed by the same investigator, using the same needle and toxin obtained from one vial from each brand. After the reconstitution of the toxins with saline, vials were maintained at 4°C for no longer than 4 hr. Three days after surgery, the expected time for the toxin to exert its maximum effect on SNAP-25 in rodents,[17] animals were euthanized and their bladders were collected.

Tissue Processing

All bladders were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6 hr, stored overnight in 30% sucrose in 0.1 M phosphate buffer and then divided into five segments; dome, upper, middle and lower body, and trigone. Serial transverse 20 μm sections from each segment were cut in a cryostat, collected on Superfost Plus slides and stored at −20°C until further processing. Detection of cSNAP-25 immunoreactivity was performed by the ABC method.[18] Slices were dehydrated with solutions of increased concentration of ethanol, cleared with xylene, mounted with Eukitt, and coverslipped.

Image and Statistical Analysis

Five randomly collected sections from each bladder segment (dome, upper, middle and lower body, and trigone; Fig. 1A) were photographed in a Zeiss Axioscope 40 microscope using the AxioVision 4.6 software. Using Adobe Photoshop CC software, images were mounted to create full figures of the five bladder sections and IR cSNAP-25 fibers were hand drawn and counted. The entire process was done blindly to the treatment groups. After counting IR fibers in the 24 animals the mean number of fibers/section was calculated for each brand and compared using a t test from the GraphPad Prism software ($P < 0.05$ statistically significant).

RESULTS

Expression of Cleaved SNAP-25 After OnabotulinumtoxinA or AbobotulinumtoxinA Administration in the Bladder Wall

Three days after a single injection of 0.5 U of onabotA or abobotA at the top of the bladder dome, cSNAP-25 immunoreactivity was observed in all the sections analyzed from each bladder segment, dome, upper, middle and lower body, and trigone (Fig. 1). IR fibers were generally more abundant in sections obtained from the lower body and trigone (Fig. 1A, C, and D). Immunoreaction was observed in the mucosa and in the muscular layers (Fig. 1C and D). In saline injected animals, no immunoreactivity was observed.

Relative Potency of OnabotulinumtoxinA and AbobotulinumtoxinA After Administration in the Bladder Wall

The average number of cSNAP-25 IR fibers per section was 341 ± 301 in the group treated with onabotA and 208 ± 152 in those treated with abobotA (Fig. 2). This difference was statistically significant (0.003). This suggests that onabotA is more potent than abobotA, with a conversion ratio of 1 U of onabotA to 1.6 U of abobotA.

Variation of the Expression of Cleaved SNAP-25 After OnabotulinumtoxinA or AbobotulinumtoxinA Administration in the Bladder Wall

The total number of cSNAP-25 positive fibers/bladder varied markedly in each experimental group. In the onabotA group, the four animals with stronger cSNAP-25 immunoreactivity had a total of $3{,}065 \pm 302$ positive fibers in the five sections. On the other hand, the four animals with less labeling had only 578 ± 106 positive fibers in the five bladder sections (Fig. 3A). This resulted in a fivefold variation between injections. In the abobotA group, the four animals with stronger IR had $1{,}628 \pm 326$ and the four animals with lower IR had 502 ± 105 positive nerves, representing a threefold variation (Fig. 3B).

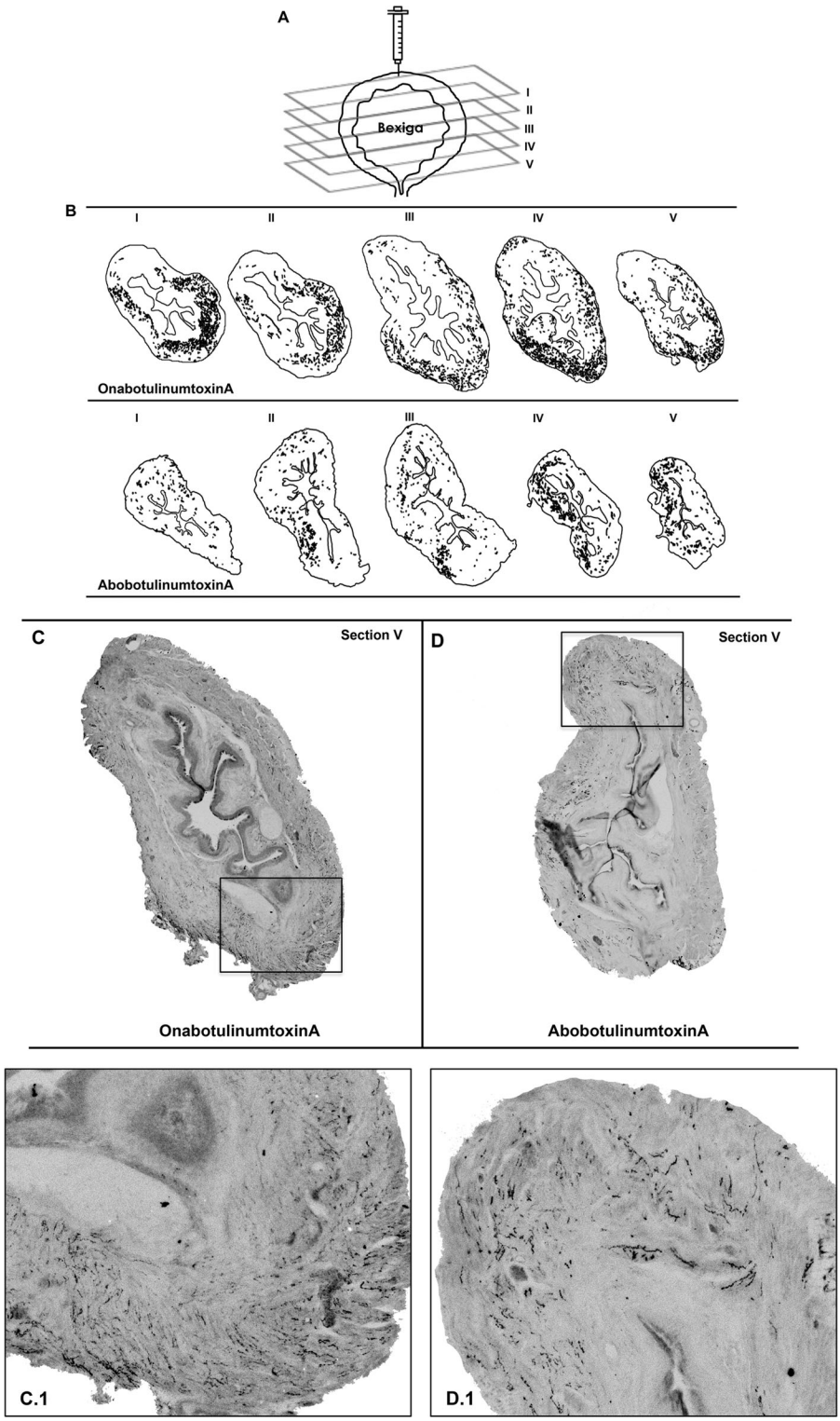

Fig. 1. **A**: Schematic representation of the five sections of the bladder considered for the quantification of cleaved synaptossomal-associated protein of 25 kDa (cSNAP-25). From the top to the bottom there are represented the dome, the upper, middle and lower body, and the trigone sections. **B**: Distribution of cSNAP-25 immunoreactive (IR) fibers (dark lines) in the bladder of two representative animals injected in the upper region of the bladder dome with 0.5 U of onabotulinumtoxinA (onabotA) or abobotulinumtoxinA (abobotA). From the left to the right are represented five sections taken from the dome to the trigone. **C**,**D**: Photomontages of the pictures taken from the trigonal bladder sections (section V), which gave origin to the schematic representations in image B. **C.1,D.1**: Expansion of the areas delimited by the rectangles in images C and D. cSNAp-25 immunostaining is observed as black lines.

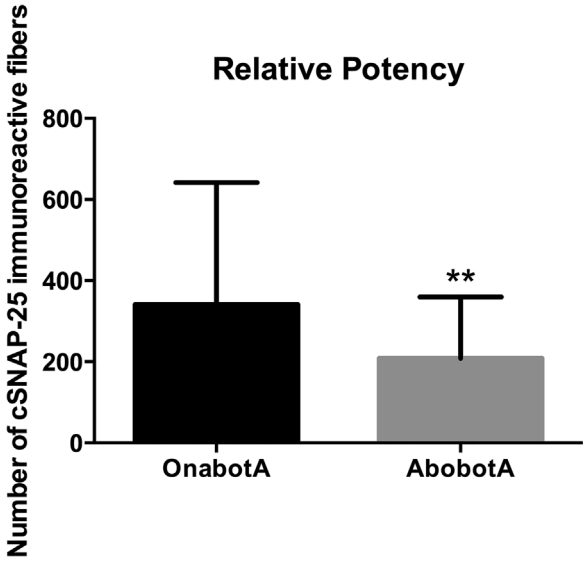

Fig. 2. Average number of cleaved synaptosomal-associated protein of 25 kDa (cSNAP-25) immunoreactive (IR) fibers in the bladder (60 sections from the 12 animals of each experimental group) of animals injected with 0.5 U of onabotulinumtoxinA (onabotA) and abobotulinumtoxinA (abobotA). The differences have statistical significance ($^{**}P < 0.01$; *t* test). Error bars show standard deviation.

DISCUSSION

In clinical practice, onabotA and abobotA are frequently used for the treatment of NDO and OAB despite the fact that only the former has been through regulatory trials.[1,2] Bioequivalence between the two brands is impossible to establish at the moment, mainly due to the fact that the potency determination of onabotA uses a biochemical cell assay based on the expression of cSNAP-25, whereas abobotA follows the classical mice LD50 assay.[11] Thus, the conversion ratio applied for bladder use is empirically determined based on skeletal muscle application, 1 U of onabotA to 3–5 U of abobotA.[10,14,19]

Our study allowed us to compare the number of cSNAP-25 IR fibers present in the mouse bladder under controlled conditions. The number of positive cSNAP-25 fibers found/bladder section after the administration of 0.5 U of onabotA was 1.6 times superior than that found for 0.5 units of abobotA. Thus, the ideal conversion ratio should be substantially lower than that currently reported in clinical practice. These results are in agreement with a recent study performed in a rat model of NDO where a conversion ratio of onabotA and abobotA of 1:1.3 U was extrapolated, using cystometric parameters as outcome.[20] Higher conversion ratios used in some OAB studies, like 1:2.5 U, may explain why the proportion of urinary retentions in patients treated with abobotA (42%) was much higher than that observed in patients who received onabotA (23%).[14]

Another aspect to take into account is the spread capacity of the two toxin formulations, which is dependent on two phenomena. One is the movement of the fluid across the bladder tissues, which is directly related to the volume of injection.[17] As the volume was the same in all experiments, this factor can be disregarded as a source of variation between the experimental groups. The second phenomenon is the diffusion, which follows a concentration gradient and may vary due to differences in formulations. In our experiments, the higher molecular weight of onabotA did not limit the spread across the bladder wall once we found immunoreactivity against cSNAP-25 in all bladder sections. The higher number of cSNAP-25 in the lower bladder sections, which are the most distant from the injection point, ought to be explained by the fact that the trigone contains the highest density of nerve fibers.[7,17]

The present study showed that a single injection of the same number of units of onabotA and abobotA in the same concentration might induce the cleavage of SNAP-25 in a remarkably different number of fibers. As a matter of fact, we observed that the number of cSNAP-25 positive fibers could vary three to five-fold, despite the fact that the needle used for injection, the location of the injection and the operator were the same in all experiments. Possible variations due to the reconstitution process of the toxin were prevented by injecting a whole set of animals using the same vial of each brand. Translating this finding into clinical practice, it seems clear that cSNAP-25 obtained after bladder wall injection of BoNT/A may have profound variations that ought to be attributed to the injection technique. The recommended administration method for neurogenic and idiopathic detrusor overactivity requires 30 and 20 injections respectively,[3] which may attenuate injection variations. Nevertheless, a careful administration of botulinum

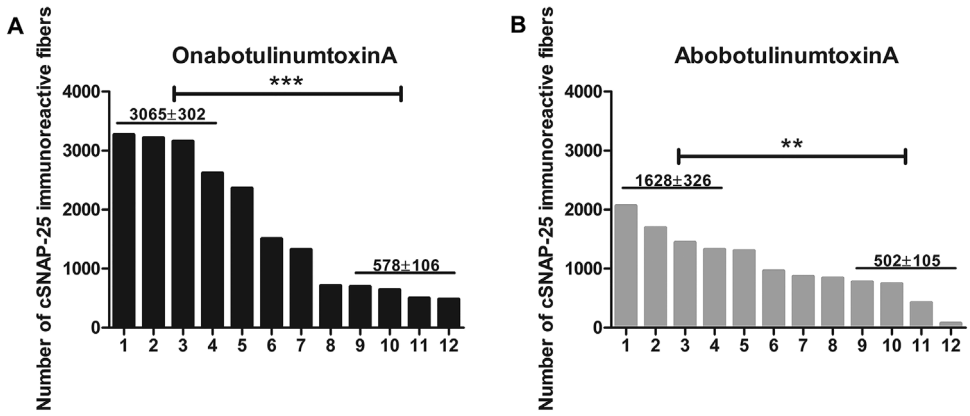

Fig. 3. **A**: Total number of cleaved synaptosomal-associated protein of 25 kDa (cSNAP-25) immunoreactive (IR) fibers counted in the bladder of each animal injected with 0.5 U of onabotulinumtoxinA (onabotA; n = 12). **B**: Total number of cSNAP-25 IR fibers counted in the bladder of each animal injected with 0.5 U of abobotulinumtoxinA (abobotA; n = 12). On top of the horizontal lines are represented the average of cSNAP-25 IR fibers and the correspondent standard deviations calculated for the four animals with the highest and lowest number of IR fibers. The differences have statistical significance ($^{***}P < 0.001$; $^{**}P < 0.01$).

toxin is a fundamental step to obtain consistently good therapeutic results.[21] This aspect was recently highlighted in a subanalysis of phase 3 regulatory trials carried out in patients with NDO. Patients without a positive response after the initial onabotA injections had excellent responses in subsequent treatments.[8]

One limitation of our study is that it was not carried out in human tissue, which for obvious ethical reasons is impossible to perform. Nevertheless, we chose mice because the bladder innervation of this species is very similar to the human one. In fact, both have the parasympathetic ganglia embedded in the bladder wall, in contrast with rat.[22] Another limitation is the fact that mice have a bladder wall much thinner than that of humans, which can increase the variability of the injections in mice, and decrease it in humans. Additionally, it is impossible to exclude the loss of toxin during the injection procedure and its contribution to the variability of results. However, it is also impossible to predict the occurrence of toxin backflow during bladder injections in patients. Therefore, our results strongly support the maintenance of injection protocols with a high number of injection points, in order to decrease variability. Another limitation can be ascribed to the method of counting fibers in the bladder wall. However, fibers were counted by the same person, in a blind way, in digitalized images. This approach circumvented the potential error that could arise from counting the nerve fibers directly through the microscope oculars.

CONCLUSIONS

In conclusion, we found that the relative potency of onabotA to cleave SNAP-25 in mice is 1.6 times higher than that of abobotA, indicating that higher conversion ratios may be excessive for bladder application. Moreover, we found strong fluctuations in the amount of cSNAP-25 that resulted from one injection of onabotA or abobotA, suggesting that the spread of the toxin from the injection point is highly variable. When translating this observation into clinical practice, a high number of bladder injections may be preferable if this risk is to be overcome.

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Publication II

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Research Paper

Impairment of sensory afferents by intrathecal administration of botulinum toxin A improves neurogenic detrusor overactivity in chronic spinal cord injured rats

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ABSTRACT

Spinal cord injury (SCI) often leads to neurogenic detrusor overactivity (NDO) due to sprouting of sensory afferents on the lumbosacral spinal cord. NDO is characterized by high frequency of voiding contractions and increased intravesical pressure that may lead to urinary incontinence. The latter has been described as one of the consequences of SCI that mostly decreases quality of life. Bladder wall injections of botulinum toxin A (Onabot/A) are an effective option to manage NDO. The toxin strongly impairs parasympathetic and sensory fibres coursing the bladder wall. However the robust parasympathetic inhibition may inhibit voiding contractions and cause urinary retention in patients that retain voluntary voiding. Here, we hypothesised that by restricting the toxin activity to sensory fibres we can improve NDO without impairing voiding contractions.

In the present work, we assessed the effect of Onabot/A on sensory neurons in chronic (4 weeks) SCI rats by injecting the toxin intrathecally (IT), at lumbosacral spinal cord level. This route of administration was shown before to have an effect on bladder pain and contractility in an animal model of bladder inflammation. We found that IT Onabot/A led to a significant reduction in the frequency of expulsive contractions and a normalization of bladder basal pressure while maintaining voiding contractions of normal amplitude. Cleavage of SNAP-25 protein occurred mainly at the dorsal horn regions where most of the bladder afferents end. Cleaved SNAP-25 was not detected in motor or preganglionic parasympathetic neurons. A significant decrease in CGRP expression, a peptide exclusively present in sensory fibres in the spinal cord, occurred at the L5/L6 segments and associated dorsal root ganglia (DRG) after Onabot/A injection in SCI animals. Onabot/A strongly increased the expression of ATF3, a marker of neuronal stress, in L5/L6 DRG neurons.

Taken together, our results suggest that IT Onabot/A has a predominant effect on bladder sensory fibres, and that such effect is enough to control NDO following chronic SCI. The mechanism of action of Onabot/A includes not only the cleavage of SNAP-25 in sensory terminals but also impairment of basic cellular machinery in the cell body of sensory neurons.

1. Introduction

The incidence and prevalence of spinal cord injury (SCI) are difficult to estimate on a yearly basis but it is assumed that >2 million people worldwide live with SCI (Lee et al., 2014). Despite recent advances, SCI is still a highly debilitating condition. Depending on the magnitude of SCI, affected individuals may present complete or incomplete loss of sensory and motor functions. With adequate treatment, certain neurological problems may be overcome, at least partially, during the first year after SCI, and patients with incomplete cord sectioning recover some limb function (French et al., 2010). A critical aspect for SCI patients is loss of voluntary control over bladder function. As a matter of fact, urinary incontinence, due to development of neurogenic detrusor overactivity (NDO), is reported by most SCI patients and represents the major cause for a decreased quality of life (Simpson et al., 2012). Indeed, after improving motor and sensory function, regaining bladder control is the highest

Abbreviations: ATF3, activating transcription factor 3; CGRP, calcitonin gene-related peptide; cSNAP-25, cleaved SNAP-25; DRG, dorsal root ganglia; IT, intrathecal; NDO, neurogenic detrusor overactivity; Onabot/A, OnabotulinumtoxinA; SCI, spinal cord injury.

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priority for SCI patients (Collinger et al., 2013; French et al., 2010; Simpson et al., 2012), due to its heavy impact on social engagement and performance of daily tasks.

Experimental studies have shown that bladder C-fibres play a fundamental role in the appearance of NDO. De Groat and co-workers demonstrated that after chronic SCI in cats, NDO emergence could be abolished by the administration of systemic capsaicin (Cheng et al., 1995, 1999). These data suggested that after SCI, the synaptic reorganization responsible for the new abnormal spinal micturition reflex is mediated by C-fibres (de Groat et al., 1990). In chronic SCI rats, the sprouting of peptidergic fibres around the preganglionic parasympathetic neurons of the lumbosacral spinal cord confirmed the role of C-fibres in the previous physiological observations (Zhang et al., 2008). Accordingly, the impairment of C-fibres has been attempted for long time to control NDO. Intravesical administration of capsaicin and resiniferatoxin (RTX) has been assessed in pilot clinical trials with considerable success but unfortunately, were not followed by appropriate regulatory trials (Cruz et al., 1997; Fowler et al., 1994).

Current management of NDO include bladder injections of OnabotulinumtoxinA (Onabot/A) (Cruz et al., 2011; Kennelly et al., 2015; Schurch et al., 2000). Successful results rely on the toxin's ability to block neuroexocytosis, by cleaving the SNAP-25 protein required for the accurate assembly of synaptic vesicles with the pre-synaptic cell membrane (Chancellor et al., 2008). Thus, the detection of cleaved SNAP-25 (cSNAP-25) has been used as a reliable marker of Onabot/A activity both in peripheral and central nervous systems (Coelho et al., 2012a, 2012b, 2014; Oliveira et al., 2015). Onabot/A is a non-specific neurotoxin that impairs almost all motor but only half of sensory fibres coursing the bladder wall (Coelho et al., 2010, 2012a, 2012b). This dual effect may certainly contribute to the high cure rates of urinary incontinence and strong reduction in the maximal detrusor pressure obtained with this toxin in patients with NDO of spinal origin. However, in patients who void spontaneously the predominant action on parasympathetic fibres is also responsible for a high rate of urinary retention, forcing patients to initiate intermittent self-catheterization. (Cruz et al., 2011; Cruz and Nitti, 2014; Ginsberg et al., 2013).

It is accepted that an important step ahead in botulinum toxin field would be the development of a toxin that specifically targets sensory fibres. In this way one expects to control NDO while minimizing the risk of urinary retention as observed in the pilot studies with capsaicin or RTX. However, since modified toxins are not yet available, testing such hypothesis for botulinum toxin A requires a method of administration that only targets sensory fibres. In a recent study, we demonstrated a reduction in abdominal pain and bladder hyperactivity in a model of chronic bladder inflammation after intrathecal (IT) administration of botulinum toxin at L5/L6 spinal cord level (Coelho et al., 2014a, 2014b). Cleaved SNAP-25 was concentrated between L4 and L6 spinal cord segments and none could be seen on motor neurons or in preganglionic parasympathetic neurons of the intermediolateral gray matter. Accordingly, no locomotion deficits were observed in the injected animals and the ability of the bladder to contract and empty completely at voiding remained intact. Thus, in the present study we evaluated the predominant sensory effect of intrathecal administration of botulinum toxin on the bladder function of chronic SCI rats and investigate putative underlying mechanisms of action.

2. Methods

2.1. Animals

All experiments were performed in female Wistar rats (Charles River, France) weighing 220–250 g that were maintained under a 12 h light/12 h dark schedule, with free access to food and water. Experimental procedures were carried out according to the European Communities Council Directive 2010/63/EU. All efforts were done to reduce the number of animals used and their suffering.

2.2. Reagents and drugs

Surgeries were performed under deep anaesthesia induced by an intraperitoneal (i.p.) injection of a mixture of ketamine (6 mg/100 g of body weight) and medetomidine (0.025 mg/100 g of body weight) diluted in saline. For cystometries and terminal handling animals received a subcutaneous (s.c.) injection of urethane (1.2 g/kg of body weight). OnabotulinumtoxinA (Onabot/A) was purchased from Allergan, CA, USA. The rabbit polyclonal cSNAP-25 antibody raised against the truncated C-terminal peptide of SNAP-25, was a kind gift from Ornella Rossetto and has been previously described (Antonucci et al., 2008; Coelho et al., 2014a, 2014b). Mouse monoclonal calcitonin gene-related peptide [(CGRP) (ab81887)], rabbit polyclonal β-actin (ab8227) and mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245) antibodies were obtained from Abcam, Cambridge, UK. Rabbit polyclonal activating transcription factor 3 (ATF3) (SC-188) and HRP-labelled secondary antibodies were purchased from Santa Cruz Biotechnology, Germany. Alexa™ 568 and 488 fluorochrome-labelled secondary antibodies, produced in donkey, were obtained from Molecular Probes Europe, Life Technologies, UK.

2.3. Spinal cord transection and catheter implantation

The model of chronic SCI induced by a complete spinal cord transection at the level of low thoracic spinal segments T8/T9 was chosen for the present study (Cruz et al., 2006; de Groat et al., 1990; Frias et al., 2015). Animals were divided into four experimental groups: (A) spinal intact + saline IT (n = 8); (B) spinal intact + Onabot/A IT (n = 8); (C) SCI + saline IT (n = 10); (D) SCI + Onabot/A IT (n = 10). Groups A and B were used as sham-operated controls, in which the spinal cord was exposed but not sectioned. Groups C and D were submitted to SCI. All animals underwent surgical implantation of a sterile silicone catheter (SF Medical; internal diameter: 0.3 mm, outer diameter: 0.635 mm) (Coelho et al., 2014a, 2014b). Briefly, under deep anaesthesia animals were submitted to laminectomy at T8/T9 level. The meninges were pierced and a silicone catheter was inserted in the subarachnoid space and pushed until the tip reached the L5/L6 spinal cord segment. The other tip was sealed and placed subcutaneously at the back of the neck for drug delivery 4 weeks after surgery (saline for groups A and C or Onabot/A for groups B and D). Catheter placement was followed by complete sectioning of the spinal cord in groups C and D and haemostatic sponge was placed between the retreated ends of the cord. Animals were left to recover and carefully monitored, receiving a daily i.p. injection of antibiotic (ciprofloxacin, 1 mg/kg) for 10 days after surgery. Bladders were manually emptied by abdominal compression to avoid urinary retention, twice daily for a period of 2 weeks. Female rats were preferred due of the difficulty to manage urinary retention in male rats.

2.4. IT drug delivery and cystometries

Four weeks after surgery, the catheter tip at the animal back was externalized and saline or Onabot/A (5 U diluted in 50 μL of saline) were delivered. Three days later, animals underwent cystometry. Animals were anaesthetized (urethane 1.2 g/kg, subcutaneous injection) and body temperature was maintained at 37 °C with a heating pad. Bladders were exposed via a midline low abdominal laparotomy. A 21-gauge needle connected to an infusion pump and to a pressure transducer was inserted into the bladder dome. Animals were left untouched for 15–30 min to allow bladder stabilization, after which saline infusion was initiated (constant rate of 6 mL/h), while intraluminal pressure was measure and recorded for 1 h. The urethra remained

unobstructed during cystometric recording so that infused saline could easily be expelled during bladder contractions. Cystometrograms were analysed and frequency, amplitude and peak and basal pressure of bladder reflex contractions were quantified using the LabScribe software (iWorx, World Precision Instruments).

2.5. Tissue processing for immunohistochemistry

After cystometries, experimental groups were divided. Half of the animals from each group were perfused through the ascending aorta with calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2 \cdot H_2O$, 0.4 mM $MgSO_4 \cdot H_2O$, 1.2 mM $NaH_2PO_4 \cdot H_2O$, 5.5 mM glucose and 26.2 mM $NaHCO_3$) followed by cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Before tissue collection, the position of the IT catheter was verified and only the animals in which the catheter was correctly placed were considered for further analysis. The spinal cord segments L5 and L6 and respective dorsal root ganglia (DRG) were collected, post-fixed for 4 h in 4% PFA and cryoprotected overnight in 30% sucrose in PB with 0.1% sodium azide. Transverse serial sections (20 μm for spinal cord segments and 10 μm for DRG) were obtained in a Leica cryostat, collected in Superfrost Plus slides and stored at −20 °C until further processing.

For immunofluorescence analysis, alternate slides were thawed, washed with saline 0.1 M phosphate buffer (PBS) and PBS containing 0.3% Triton X-100 (PBST) and blocked with 10% of normal horse serum (NHS) in PBST for 1 h. Primary antibodies (anti-cSNAP-25 1:1000; anti-CGRP 1:8000; anti-ATF3 1:200) were diluted in 2% NHS in PBST for 48 h at 4 °C. After several washes with PBST, sections were incubated with species-specific Alexa™ fluorochrome-labelled secondary antibodies for 1 h at room temperature. After several washes with PBST and PBS, sections were mounted with an anti-fade mounting medium (Thermo Fischer Scientific, MA, USA) and representative images were obtained using a Zeiss microscope (Axioimager Z1, Zeiss Z1 from Zeiss, Oberkochen, Germany) using the Axiovision 4.8 software. The intensity of CGRP immunoreactivity in the spinal cord was quantified by densitometry using the Fiji software (Schindelin et al., 2012). Staining intensity was averaged from 15 to 20 spinal sections per animal by selecting the dorsal horn area exhibiting CGRP immunoreaction (laminae I, II, III and IV), and after deduction of background intensity. In DRG, the number of nuclei positively labelled for ATF3 was averaged after counted using the Fiji software. In both cases, data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant.

2.6. Tissue processing for western blotting

After cystometries half of the animals from each experimental group were euthanized and L5/L6 DRG were collected and immediately stored at −80 °C until further processing. Tissue was homogenized using a lysis buffer (Tris 50 nM, NaCl 150 nM, Triton X-100 0.5%, EDTA 1 nM, pH 7.6) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Homogenates were sonicated and the protein concentration determined using the Bradford assay (Biorad, California, USA). Thirty μg of tissue homogenates were separated on a 12% polyacrylamide gel using and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk in Tris buffer saline with 0.1% Tween-20 (TBST) and incubated with anti-CGRP 1:8000 in 5% bovine serum albumin (BSA) in TBST overnight at 4 °C. Blots were then incubated with anti-mouse HRP-conjugated antibody (1:5000) and β-actin (1:5000) or GAPDH (1:10,000) for 1 h at room temperature. The bound antibodies were detected by chemiluminescence (Supersignal West Pico Chemiluminescence Substrate, Pierce). Digital images were obtained using a Chemidoc MP System Imager and the ImageLab 5.1 software (Biorad). Membranes were then stripped by incubating membranes with 10% SDS for 30 min at room temperature. Following a second blockade, membranes were re-incubated with anti-ATF3 1:200 overnight at 4 °C. Signal detection was performed as previously described. Digital images of western-blots were obtained and protein levels of CGRP and ATF3 were quantified by densitometry and normalized against beta-actin or GAPDH using the Fiji software (Schindelin et al., 2012).

2.7. Data analysis

All data was statistically analysed using the one-way ANOVA followed by the Tukey's multiple comparison test using the GraphPad Prism 6 software. Data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Intrathecal administration of Onabot/A improves bladder function after spinal cord transection

Spinal intact animals that received IT saline exhibited normal bladder reflex activity, characterized by regular contractile activity during cystometry with an urinary frequency of 0.5 ± 0.1 contractions/min, an amplitude of voiding contractions of 31.9 ± 4.3 cm H_2O and a basal pressure of 8.6 ± 3.1 cm H_2O (Fig. 1A). The administration of Onabot/A IT did not cause any alteration in bladder contractions. Hence, no changes of cystometric parameters were observed (Fig. 1B). In these animals, frequency and amplitude of voiding contractions were 0.5 ± 0.1 contractions/min and 32.6 ± 2.9 cm H_2O, respectively, and basal pressure was 4.5 ± 2.0 cm H_2O (Fig. 1, graphs A to C).

In SCI animals, the typical abnormal pattern of bladder reflex activity, urodynamically designated as NDO, was evident 4 weeks after surgery (Fig. 1C). At this time, the frequency of bladder contractions was significantly increased to 2.0 ± 0.8 contractions/min ($p < 0.001$ versus spinal intact). The amplitude of these reflex contractions was significantly decreased to 14.9 ± 9.5 cm H_2O ($p < 0.05$ versus spinal intact), while basal pressure significantly increased to 31.7 ± 13.1 cm H_2O ($p < 0.01$ versus spinal intact) (Fig. 1, graphs A to C). Administration of saline IT did not induce any alteration of bladder activity in SCI rats.

In contrast with saline, IT administration of Onabot/A 4 weeks after SCI markedly changed bladder function (Fig. 1D). The toxin significantly decreased the frequency of expulsive bladder contractions (0.9 ± 0.3 contractions/min). In addition, voiding contractions maintained normal amplitude and could empty the bladder. Bladder baseline pressure was normalized (31.2 ± 12.0 cm H_2O and 14.3 ± 7.8 cm H_2O, respectively, Fig. 1, graphs).

As in previous studies (Coelho et al., 2014a, 2014b), urinary retention, other visceral side effects, motor impairment, worsening of limb paralysis (in SCI animals) or respiratory depression were never observed in any of the spinal intact and SCI animals receiving IT Onabot/A.

3.2. Intrathecal administration of Onabot/A cleaves SNAP-25 on lumbosacral spinal cord neurons

At the L5 and L6 spinal cord segments, where the majority of central projections from peptidergic sensory neurons that innervate the urinary bladder are located (Honda, 1985; Morgan et al., 1981; Nadelhaft and Booth, 1984), strong cSNAP-25 immunoreactivity was found in animals treated with IT Onabot/A (Fig. 2). The immunoreactive fibres were mainly observed coursing laminae I and II of the dorsal horns (Fig. 2A) and also detected in the proximity of motor neurons' cell bodies in the ventral horns (Fig. 2C). The cleaved form of SNAP-25 was also found, but to a much lower intensity, in spinal cord segments at the vicinity of the administration area, such as L4 and S1. Motor or parasympathetic

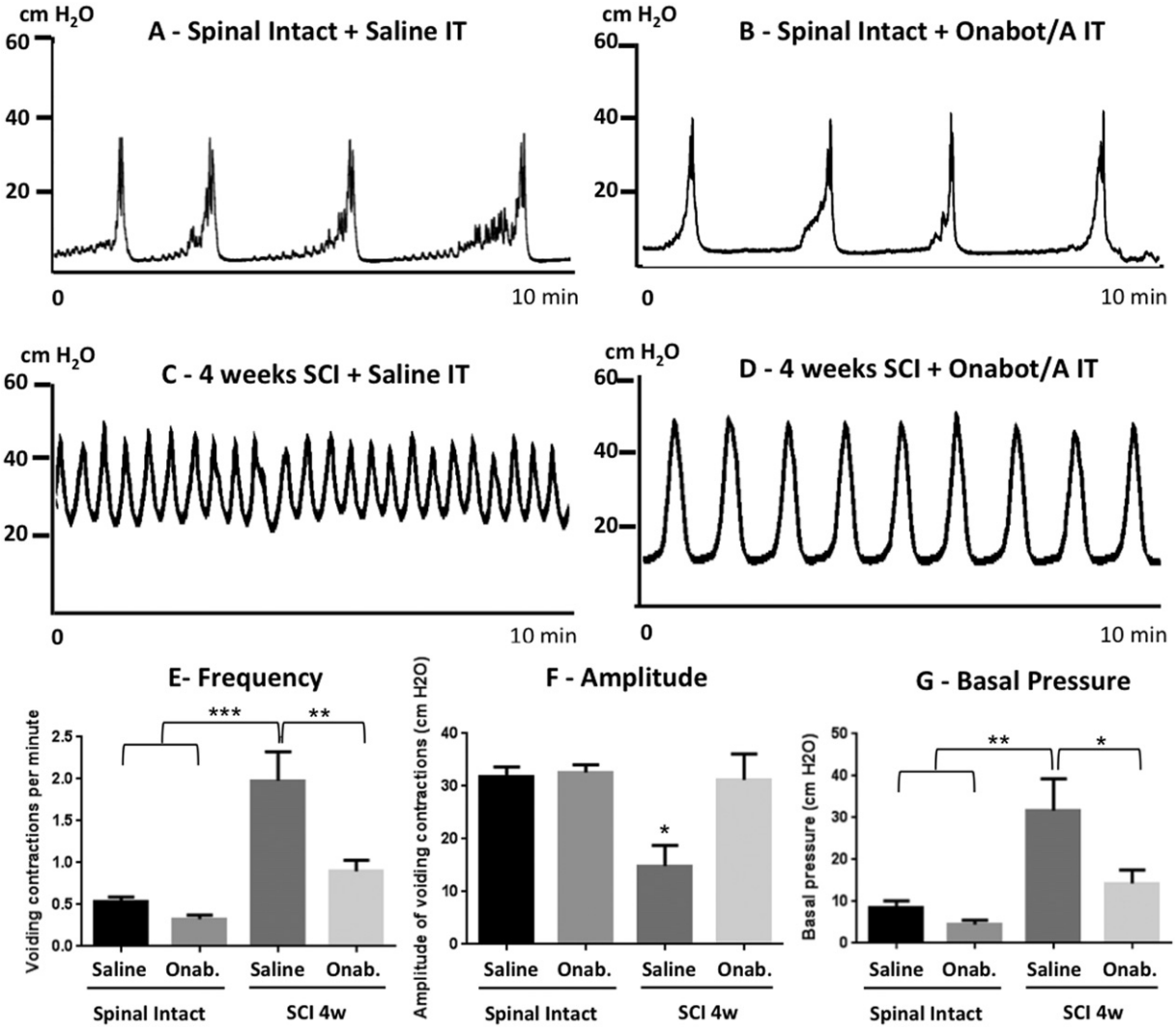

Fig. 1. Cystometrograms depicting the voiding pattern of all experimental groups. In spinal intact animals, irrespective of IT administration of saline or Onabot/A, a normal voiding pattern is observed (A and B, respectively). In SCI animals that received saline, a typical pattern of NDO is well established (C). SCI animals treated with Onabot/A showed an improved voiding pattern with significantly reduced urinary frequency and basal pressure and increased amplitude of voiding contractions (D). Analysis of urodynamic parameters such as frequency of voiding contractions (E), amplitude of voiding contractions (F) and basal pressure (G) are detailed in the graphs. Graphs represent the average ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$; one-way ANOVA followed by Newman-Keuls multiple comparison test.

preganglionic nerves in the intermediolateral nucleus (IML) did not express cleaved SNAP-25 (Fig. 2B).

3.3. Intrathecal administration of Onabot/A alters the expression of CGRP at the spinal cord after spinal cord injury

In spinal intact animals the classic bilateral dorsal horn distribution of CGRP-positive processes was observed in the L5 and L6 segments of the spinal cord. CGRP immunoreactivity was primarily detected in nerve endings coursing in the superficial laminae (I and II) and less abundantly in lamina X (Fig. 3A). Intrathecal administration of Onabot/A in spinal intact animals did not change the intensity of CGRP immunoreaction (Fig. 3B). In spinal sections from SCI animals, CGRP immunoreaction was more intense and the CGRP-positive processes were longer with immunoreactive terminals penetrating laminae III and IV and abundantly detected in lamina X and Lissauer's tract (Fig. 3C; $p < 0.005$ versus spinal intact). Intrathecal administration of Onabot/A 4 weeks after SCI reduced the intensity of CGRP immunoreactivity to values comparable to those observed in spinal intact animals (Fig. 3D).

3.4. Intrathecal administration of Onabot/A causes neuronal changes at the dorsal root ganglia

In spinal intact animals, CGRP was present in the cytoplasm of small diameter neurons of the L5 and L6 DRG. Intrathecal administration of Onabot/A to these animals appeared to decrease the number of DRG neurons expressing CGRP (Fig. 4). To quantify this qualitative change western blot was performed to assess changes of CGRP levels in the DRG. In SCI animals, CGRP expression increased 302% ($p < 0.005$) in comparison with spinal intact rats. Intrathecal injection of Onabot/A delivered to SCI animals, significantly reduced CGRP expression to control levels (Fig. 4).

We also analysed the expression of ATF3, a nuclear marker of neuronal stress (Chen et al., 1996; Tsujino et al., 2000). Immunostaining of DRG sections identified ATF3 presence in neuronal nuclei of all experimental groups (Fig. 5). A qualitative inspection of those DRG sections suggested a substantial increase of ATF3 in SCI animals treated with IT Onabot/A (Fig. 5A and B). Quantification of ATF3 positively-labelled nuclei in L5 and L6 DRG sections showed that in spinal intact animals the number of nuclei was 0.5 ± 0.1 and IT administration of Onabot/A

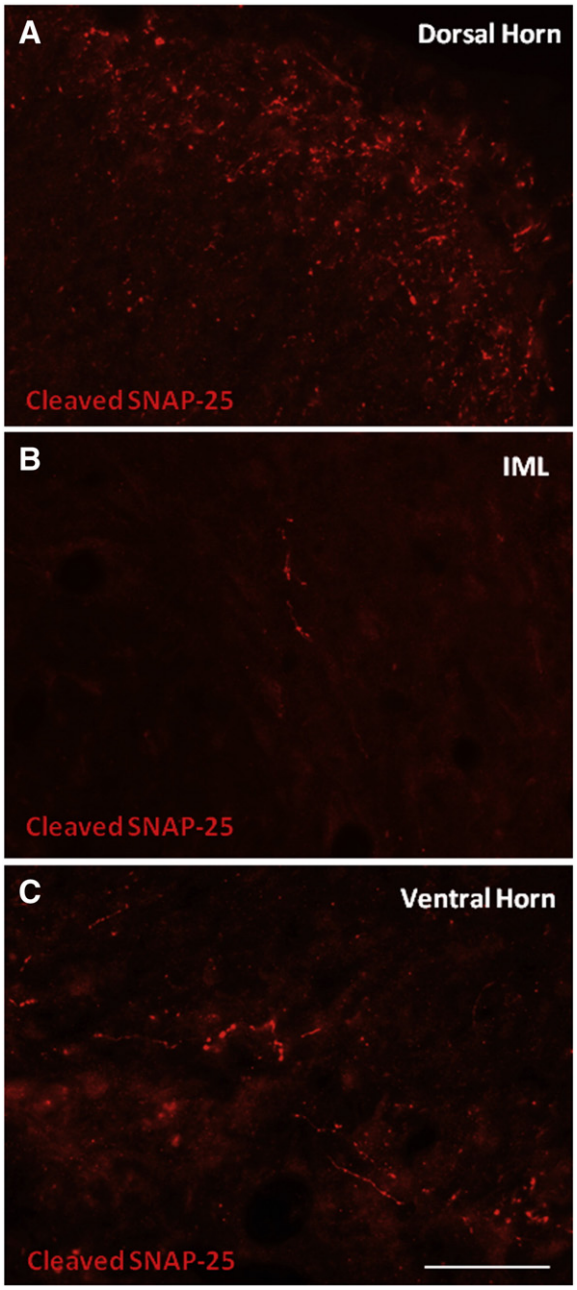

Fig. 2. Immunohistochemistry for cleaved SNAP-25 in the lumbosacral spinal cord. Cleaved SNAP-25 was detected by fluorescence immunohistochemistry in the L5 and L6 spinal segments after IT administration of Onabot/A. The cleaved protein was observed in the dorsal horn mainly in the superficial laminae I and II (A) and in nerve fibres around the motor neurons of the ventral horn (C). No immunoreaction was found in motor neurons of the intermediolateral gray matter (IML) (B).

did not significantly altered that number (0.853 ± 0.357) (Fig. 5D). SCI induced a significant increase of ATF3 positive nuclei (2.976 ± 0.453) and IT administration of Onabot/A in SCI animals further increased the expression of that transcription factor (5.084 ± 1.864) (Fig. 5D). Quantitative analysis of the total protein expression showed similar results (Fig. 5C and E). ATF3 was present at very low levels in DRG from spinal intact animals (Fig. 5C and E). ATF3 expression slightly increased in SCI rats treated with IT saline (178.3% compared to spinal intact) and further raised significantly (296.1% compared to spinal intact, $p < 0.05$) in SCI rats treated with IT Onabot/A (Fig. 5C and E).

4. Discussion

The main finding of the present study is that IT administration of Onabot/A causes a profound impairment of sensory fibres through a mechanism that includes SNAP-25 cleavage in terminals at the spinal cord dorsal horn as well as changes in gene transcription at the DRG neurons in chronic SCI rats. As a consequence of these changes in sensory neurons at the lumbosacral spinal cord level, a marked improvement in neurogenic bladder dysfunction was observed.

We found that IT administration of Onabot/A al lumbosacral spinal cord level improves SCI-induced NDO, as the abnormal pattern of micturition observed in rats 4 weeks after complete SCI was significantly improved by this treatment. Indeed, 48 h after toxin delivery, the frequency and baseline pressure of reflex bladder contractions were significantly decreased. Despite being IT injected, Onabot/A did not cause any case of urinary retention, excluding any remarkable impairment of the parasympathetic innervation of the bladder. Moreover, IT treated SCI rats did not show any obvious weakening of the upper limb movements when compared to saline treated SCI animals. Also, no deaths by respiratory failure occurred in the treated group confirming the absence of any relevant paralysis of the respiratory muscles. This is in agreement with our previous observations in naïve and bladder inflamed animals injected IT with Onabot/A at the same spinal cord level to treat bladder pain. Toxin treated rats maintained bladder contractility and did not develop urinary retention. Moreover, locomotion was not affected since walking distance in the open field test was similar to saline injected and sham operated rats. Reduction of pain levels was striking in animals with bladder inflammation that received IT Onabot/A (Coelho et al., 2014a, 2014b). Altogether these findings rule out a major impairment of the parasympathetic activity as the main reason for bladder improvement in SCI rats treated with IT Onabot/A.

The effect of IT Onabot/A in lumbosacral sensory endings was demonstrated in the present study. We observed intense immunoreactivity for cSNAP-25 in Onabot/A-treated rats restricted to the superficial dorsal horn of lumbosacral segments, where most sensory fibres terminate. In contrast, no cSNAP-25 was found in cell bodies of neurons located in the ventral horn or IML which might correspond to motor or preganglionic parasympathetic neurons.

The typical increase of CGRP immunoreactivity in the lumbosacral spinal cord in chronic SCI rats (Zinck et al., 2007) was also observed in the present study and substantially reduced by IT Onabot/A. One should recall here that a similar decrease in CGRP immunoreactivity was reported in our previous work on IT Onabot/A in a model of chronic bladder inflammation (Coelho et al., 2014a, 2014b). This change in CGRP expression, a peptide exclusively present in sensory terminals in the spinal cord, is an additional evidence of the strong effect of IT Onabot/A on sensory fibres. Additionally, we demonstrated that IT injected Onabot/A caused profound changes in lumbosacral DRG neurons. This was evidenced in two different ways: CGRP expression was decreased in the cell bodies of DRG neurons while there was a strong up-regulation of ATF3. The latter is an immediate-early transcription factor that encodes the transcription of genes related to the instant response to injury, survival and regeneration (Hai et al., 1999; Tsujino et al., 2000). Previous works supporting this mechanism have shown ATF3 up-regulation in the nuclei of sensory neurons following stress and traumatic injury in animal models of spinal cord compression injury, spinal hemisection and monoarthritis (Hai et al., 1999; Nascimento et al., 2011; Tsujino et al., 2000). Also, it has been shown that ATF3 is up-regulated in the human DRG after traumatic dorsal root avulsion (Linda et al., 2011). In our work, sham-operated animals expressed residual levels of ATF3, possibly reflecting the effects of surgical procedures as it has been previously observed (Huang et al., 2006). Spinal cord injured rats had a marginal increase in the number of ATF3-positive neurons, as a consequence of the

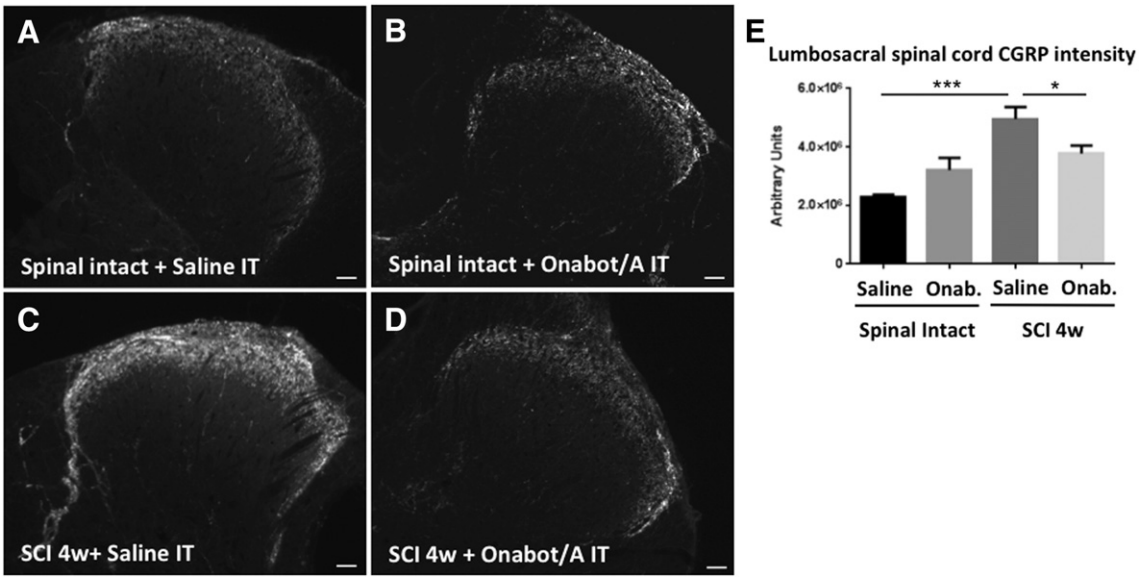

Fig. 3. Distribution of CGRP in the lumbosacral spinal cord. CGRP was detected by immunohistochemistry in the dorsal horn of the L5 and L6 spinal segments. In spinal intact animals, irrespective of the treatment with saline or Onabot/A CGRP was distributed mainly in the superficial laminae (I and II) (A and B). Four weeks after SCI, CGRP distribution increased and was observed in deeper laminae (III and IV) as well as laminae X and Lissauer's tract (C). When Onabot/A treatment was delivered to SCI animals, the distribution of CGRP was similar to the controls (D). The intensity of CGRP immunoreactivity was quantified by densitometry and average values are detailed in the graph (E). Graph columns represent average intensity per experimental group ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$; one-way ANOVA followed by Newman-Keuls multiple comparison test.

chronic state of the spinal injury. This observation was in accordance with previous works where ATF3 was significantly increased in the DRG after spinal cord compression injury (Huang et al., 2006). Surprisingly, IT Onabot/A markedly increased ATF3 in DRG cells further suggesting the sensory impairment caused by the IT toxin administration.

The increase of ATF3 shown in this work raises the possibility of another mechanism of action for Onabot/A in what concerns sensory fibres. Under the classical mechanism of action of Onabot/A on SNAP-25, we would predict that CGRP release was impaired and that this peptide would accumulate in sensory neurons. Instead, we observed a decrease of CGRP in the sensory terminals, located in the dorsal horn, and in the cell body of DRG neurons. This neuropeptide is synthesized in the soma and transported to the terminal processes coursing in laminae I–II of the spinal cord. Thus, this study suggests that Onabot/A affects sensory fibres not only at their terminals but also at their cell

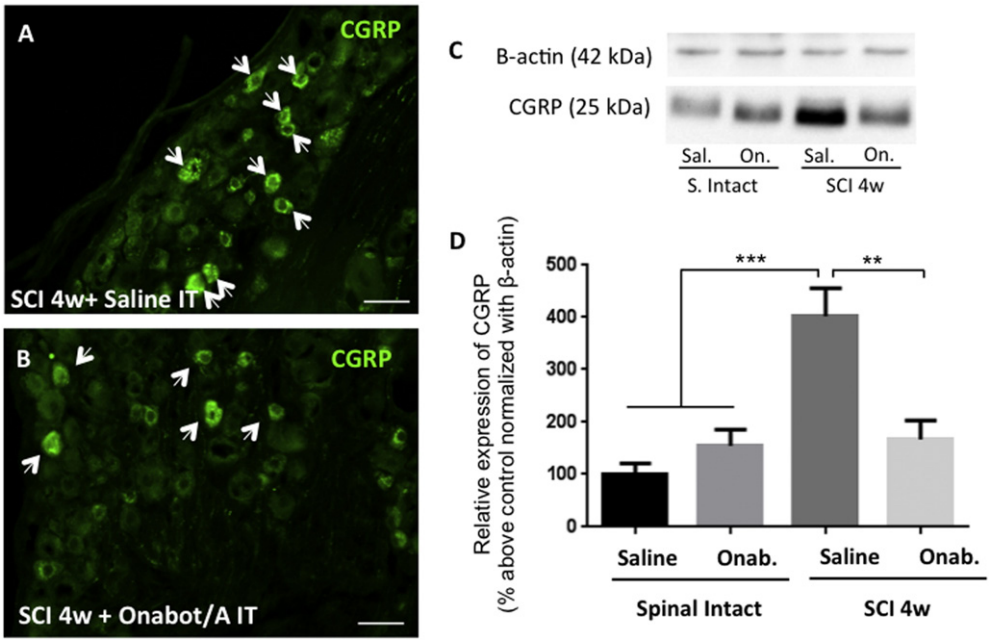

Fig. 4. Expression of CGRP in the dorsal root ganglia. CGRP expression in L5 and L6 dorsal root ganglia was detected by immunohistochemistry and quantified by western immunoblotting. (A) and (B) show CGRP immunoreaction in the L6 DRG of SCI animals treated with saline and Onabot/A, respectively. (C) represents western immunoblot detection of CGRP (25 kDa) and β-actin (42 kDa) corresponding to each experimental group (from left to right there are lanes from spinal intact + saline, spinal intact + Onabot/A, SCI 4w + saline and SCI 4w + Onabot/A). (D) represents the averages of CGRP relative expression determined for each group, quantified by western immunoblotting and normalized against β-actin. In spinal intact animals, Onabot/A did not alter the levels of CGRP expression (C, D). Four weeks after SCI, expression of CGRP in DRG increased significantly (A, C, D). After Onabot/A treatment CGRP expression decreased markedly to levels identical to those found in spinal intact animals (B, C, D). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$; one-way ANOVA followed by Newman-Keuls multiple comparison test.

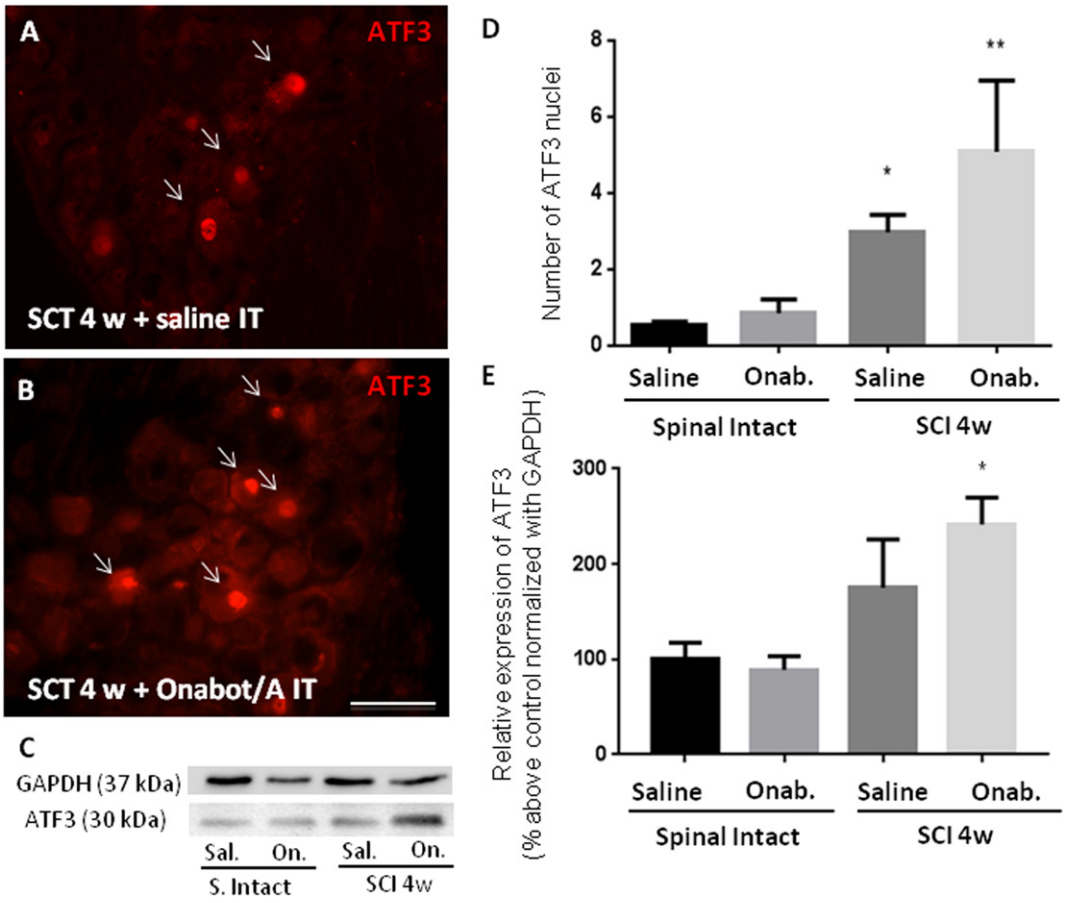

Fig. 5. Expression of ATF3 in the dorsal root ganglia. ATF3 expression in L5 and L6 DRG was detected by immunohistochemistry and quantified by western immunoblotting. In spinal intact animals ATF3 is expressed in low levels irrespective of the treatment with saline or Onabot/A. SCI lead to a significant increase of ATF3 expression levels and number of labelled nuclei (C, D, E) which further raised after Onabot/A treatment (C, D, E). (A) and (B) represent ATF3 immunoreaction at the L6 DRG of SCI animals treated with saline and Onabot/A, respectively. (C) represents western immunoblot detection of ATF3 (30 kDa) and GAPDH (37 kDa) corresponding to each experimental group (from left to right there are represented lanes from spinal intact + saline, spinal intact + Onabot/A, SCI 4w + saline and SCI 4w + Onabot/A). (D) represents the average of ATF3 positive nuclei in the L5 and L6 DRG. (E) represents the average of ATF3 relative expression determined for each group, quantified by western immunoblotting and normalized against GAPDH. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$; one-way ANOVA followed by Newman-Keuls multiple comparison test.

body, possibly impairing the cytoplasmic machinery responsible for protein synthesis, post-translational modifications, packing and/or axonal transport. Thus, it seems that the effects of Onabot/A on neurotransmission are not restricted to cleavage of synaptic proteins but may result from profound alteration of the basic neuronal metabolism. This hypothesis has been raised by other studies that showed a toxin activity in cellular mechanisms independent of SNAP-25 cleavage such as the arachidonic acid pathway, neuritogenesis, cell cycle, apoptosis and gene expression (Matak and Lackovic, 2015).

Despite the effective control of NDO with IT Onabot/A, the leading objective of this study was not to demonstrate an eventual clinical role of this route of administration for the toxin in NDO patients. Bladder wall injections of Onabot/A are highly effective to manage NDO symptoms in SCI patients, given the marked long-lasting improvement of urinary incontinence and decrease of maximal detrusor pressure seen in the clinical pivotal trials (Cruz et al., 2011; Ginsberg et al., 2012). Rather, the objective was to investigate the possibility of controlling NDO of spinal cord origin by the predominant impairment of bladder sensory fibres with botulinum toxin while sparing the parasympathetic motor innervation of the bladder. One should take in mind that when injected in the bladder wall, Onabot/A acts preferentially on parasympathetic fibres, as indicated by the presence of cSNAP-25 immunoreactivity in 85% of cholinergic fibres after toxin injection in the rat bladder (Coelho et al., 2010, 2012a, 2012b). These cholinergic fibres also belong to pre-ganglionic parasympathetic neurons (Coelho et al., 2012a, 2012b) which may indicate that synaptic transmission in parasympathetic ganglia embedded in the bladder wall is also impaired by bladder wall injections of botulinum toxin A. On the contrary, the effect of bladder wall injections on sensory fibres is very modest, only one third of sensory fibres coursing the bladder wall exhibited strong cSNAP-25 immunoreactivity (Coelho et al., 2012b). According to the findings of this study, we can expect that a future recombinant botulinum toxin with a strong specificity for sensory fibres might be used to effectively treat NDO without the risk of impairing parasympathetic-mediated bladder voiding contractions.

5. Conclusion

Our study highlights that Onabot/A has a strong sensory effect. Restriction of the toxin activity to sensory terminals after intrathecal delivery is enough to control bladder function in SCI animals. The effect of Onabot/A on sensory fibres is not restricted to the cleavage of SNAP-25 and includes the impairment of basic cytoplasmic machinery required to the synthesis of neuropeptides, protein synthesis and/or axonal trafficking.

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Unpublished Observations

Oliveira R, Chambel S, Cavaleiro H, Silva R, Cruz F, Cruz CD (2019) Effects of Early Bladder Administration of Botulinum Toxin A On Neurogenic Detrusor Overactivity Following Spinal Cord Injury.

Effects of Early Bladder Administration of Botulinum Toxin A on Neurogenic Detrusor Overactivity Following Spinal Cord Injury

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Abstract

Neurogenic detrusor overactivity (NDO) affects the great majority of spinal cord injury (SCI) patients, being in the top list of their concerns, and require rapid, safe and effective management strategies. Bladder-wall injections of OnabotulinumtoxinA (Onabot/A) emerged as a promising treatment, resulting in significant urodynamic benefits and clear improvement in patients' quality of life. Toxin-based therapy is currently initiated in patients that do not respond to antimuscarinics and in a chronic phase of the lesion, when NDO is established. Here we evaluated the possibility to attenuate NDO development if we provide treatment to SCI rats in an early phase of disease progression, during spinal shock. Our results suggest that an early intervention with Onabot/A is not effective in preventing NDO development, with SCI rats treated with the toxin presenting the urodynamic pattern of NDO 4 weeks after lesion. The analysis of bladder, L5-S1 dorsal root ganglia (DRG) and spinal cord (SC) tissue corroborate findings on bladder reflex activity, with no significant changes emerging from Onabot/A early administration. In addition, here we raised the hypothesis that the absence of toxin-mediated alterations in urodynamics and neuronal reflex inducing NDO might reflect the toxin incapacity to exert its effect on synaptosomal nerve-associated protein 25 (SNAP-25) during the period of spinal shock.

Introduction

Botulinum toxin A (BoNT/A) is the current gold-standard NDO treatment for patients that are refractory to antimuscarinic therapy [1-4]. Several studies show that intra-detrusor injections of BoNT/A result in a marked reduction in the frequency of episodes of urinary incontinence as well as improvement of several urodynamic parameters, including intravesical pressure [5, 6]. When internalized by active pre-synaptic neuronal terminals, via the synaptic vesicle glycoprotein 2 (SV2) [7], this neurotoxin specifically cleaves the synaptossome associated protein of 25 kDa (SNAP-25), a protein essential for neurotransmitter release, and effectively blocks neurotransmission. Because virtually all bladder fibres express SV2 and SNAP25 [8], intradetrusor administration of BoNT/A is able to block parasympathetic, sensory and sympathetic bladder fibres [8, 9]. The effects of bladder injection with this neurotoxin are long-lasting with little secondary effects, resulting in improved quality of life for NDO patients [5].

Management of NDO is typically initiated when urinary incontinence is already a stable fixed condition, with little chances of disappearing, and when kidney damage secondary to detrusor-sphincter-dyssynergia (DSD) may have already occurred. However, an increasing number of studies suggest that assessment of urinary dysfunction, as NDO arising after SCI, should be an early priority and therapeutic intervention should also be initiated at early time points of disease progression [10]. This is supported by studies demonstrating positive effects on bladder function of SCI patients and experimental animals were found when treatments were initiated during spinal shock. These treatments included neuromodulation and antimuscarinics [11-13]. In the present study we investigated if, like other treatments, early administration of BoNT/A would results in improved bladder function in SCI rodents.

Materials and Methods

Spinal cord injury rat model

Experiments were performed in in-house bred, 220 to 290 g female Wistar rats (Charles River, France), maintained under a 12-hour light/ dark schedule, with ad libitum access to food and water. We used the rat model of largely incomplete spinal cord transection (SCT) at the high thoracic levels of T8/T9. After anaesthesia and dorsal shaving, the T13 vertebra was identified by palpation and the T7 to T10 vertebra exposed. Following a laminectomy, the spinal segments T8/T9 were visualized and a scalpel was inserted approximately 5mm perpendicularly until the tip touched the bone. All surgeries were performed by the same experimenter to ensure reproducibility. The scalpel was removed and a small piece of sterile gelfoam was inserted to limit bleeding. Sham-operated rats were used as controls. Animals were left to recover, receiving daily ciprofloxacin (1mg/kg) for 10 days after surgery. Bladders were manually emptied daily, by abdominal compression for a period of 2 to 3 weeks.

Experimental groups and treatments

Three groups of animals (n=4/groups) were used to evaluate changes in in bladder expression of SNAP-25, GAP43, TRPV1 and VAChT associated with the progression of bladder dysfunction. Bladder tissue was collected from spinal intact (INT) and from SCT rats 1 and 4 weeks after spinal lesion (1 wk and 4 wks, respectively).

Three days after spinal injury, 10U of the BoNT/A formulation OnabotulinumtoxinA (Onabot/A; Allergan, CA), diluted in 100 uL of saline, was injected in 10 points of the bladder wall of SCT (n=6) or sham-operated rats (n=3). Control SCT (n=6) and sham animals (n=5) received only saline. Toxin dosage was selected based on previous studies demonstrating its effectiveness in chronic NDO rats [14].

Recordings of bladder reflex activity

Animals underwent cystometries 4 weeks after Onabot/A injection, a time-point after spinal injury when NDO is typically present. After deep anaesthesia induced by subcutaneous urethane (1.2 g/Kg), cystometries were performed for 1 hour with constant body temperature of 37°C, maintained with a heating pad. Briefly, following abdominal incision, a 21-gauge needle, connected to an infusion pump and a pressure transducer, was inserted into the bladder dome. Twenty minutes later saline infusion was initiated (6mL/h) and bladder reflex activity recorded. Urodynamic parameters, such as minimum and maximum bladder pressures, amplitude and frequency of bladder contractions were obtained using LabScribe software (iWorx; World Precision Instruments, UK).

Western blot analysis

Bladders were collected after cystometries from INT and SCT non-treated and treated animals. In the latter, bladders were collected and cut longitudinally in half. One portion was immediately frozen and stored at -80°C. The remaining hemi-bladder was collected in 4% paraformaldehyde (PFA), post-fixed for 5 hours and cryoprotected overnight in 30% sucrose in phosphate buffer with 0.1% sodium azide. Frozen samples were homogenized in lysis buffer (Tris 50 nM, NaCl 150 nM, Triton X-100 1%, EDTA 1 nM, pH 7.6). Fifty micrograms of protein were separated on 12% polyacrylamide gel and transferred onto a PVDF membrane. Membranes were blocked for 1 hour in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 and incubated 48 hours at 4°C with primary antibodies against SNAP-25 (Synaptic Systems 111002; 1:2000), GAP43 (Abcam ab16053; 1:200), TRPV1 (Santa Cruz Biotechnology sc-12498; 1:500) and VAChT (Synaptic Systems 139103; 1:1000), followed by incubation with specific horseradish peroxidase-conjugated antibodies. Bound antibodies were detected by chemiluminescence and

digital images obtained using a Chemidoc MP System Imager and the ImageLab 5.1 software (Bio-Rad). Membranes were re-incubated (24 hours at 4°C) with an anti-GAPDH (Abcam ab8245; 1:10000) antibody and signal detection repeated as above. Three samples per group were analysed. Protein levels were quantified by densitometry using the Fiji software. All intensities measured were normalized against GAPDH.

Immunohistochemical tissue analysis

After bladder collection, SCT treated animals were perfusion fixed through the ascending aorta with calcium-free Tyrode's solution followed by PFA 4%. Spinal L5-S1 segments and adjacent dorsal root ganglia (DRG) were collected, post-fixed for 5 hours and cryoprotected overnight in 30% sucrose in phosphate buffer with 0.1% sodium azide. Transverse spinal cord and bladder 30-µm thick sections and longitudinal DRG 12-µm thick sections were obtained in a Leica cryostat and collected in cryoprotective solution and slides, respectively. Fixed hemi-bladders were also sectioned in a Leica cryostat and 30-µm thick sections collected on slides. Alternate sections from spinal cord, bladder and DRG were thawed, thoroughly washed with PBS and PBST and then blocked with 10% of normal horse serum (NHS) in PBST for 1 hour. This was followed by a 48-hour incubation at 4°C with primary antibodies against the truncated C-terminal peptide of SNAP-25 (1:4000; gift from Ornella Rossetto [15]), GAP43 (Abcam ab16053; 1:5000), ATF3 (Santa Cruz Biotechnology sc-188; 1:300), CGRP (Abcam ab81887; 1:8000). After several washes, sections were incubated with species-specific Alexa flurocrome labelled antibodies (Invitrogen - ThermoFisher Scientific) for 1 hour at room temperature. The sections were mounted with Prolong Gold mounting medium (Molecular Probes, Porto, Portugal) and representative images were obtained in a Zeiss microscope (Axioimager Z1; Zeiss) using the Axiovision 4.8 software. In DRG, the number of nuclei positively labelled for ATF3 was averaged

after counting (25-30 sections per animal). Spinal CGRP and GAP43 immunostaining was quantified by densitometry using the Fiji software.12 Intensity was averaged from 15 to 20 spinal sections per animal by selecting the dorsal horn area exhibiting immunoreactivity (laminae I-IV), and deducting background intensity.

Statistical analysis

Statistical analyses were performed in GraphPad Prism 6 Software (GraphPad, San Diego, CA) using t-test or one-way repeated-measure analysis of variance, where appropriate, followed by the Tukey multiple comparison test. All values are presented as mean ± SD and $P<0.05$ was considered statistically significant. All intergroup comparisons were evaluated and are indicated.

Results

Changes in bladder innervation after spinal cord lesion

Western immunoblotting analysis of bladder tissue from INT and 1wk and 4wk SCT animals demonstrated a significant decrease of SNAP-25 expression 1 week after SCT ($p<0.05$ vs spinal intact), returning to normal values when NDO is established ($p<0.05$ vs SCT 1 week; **Figure 1A**). Similarly, levels of GAP43, a well-described marker of neuronal sprouting, were also decreased in bladder tissue during spinal shock ($p<0.05$ vs spinal intact; **Figure 1B**), suggesting some degree of denervation. Likewise, markers of sensory (**Figure 1C**) and parasympathetic fibres ($p<0.01$ vs INT; **Figure 1D**) decreased at 1-week post SCI and appeared to recover at 4 weeks after spinal lesion.

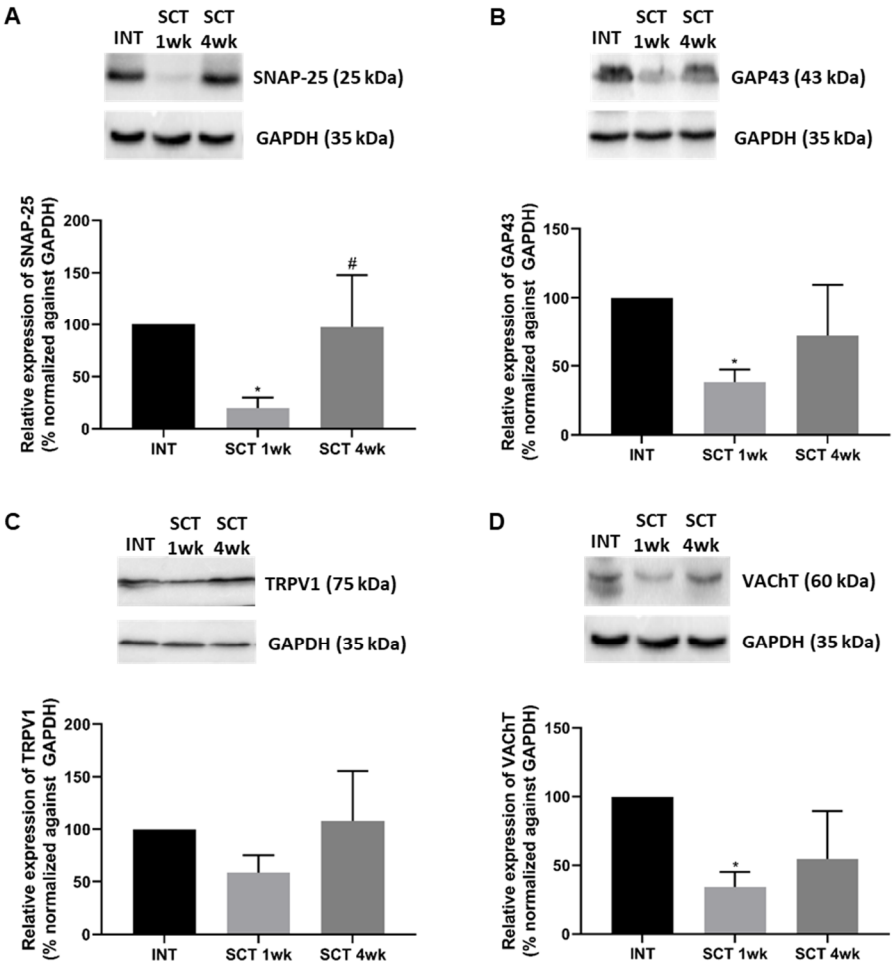

Figure 1: Changes in bladder innervation occurring after spinal cord injury. Representative Western immunoblotting bands and respective averaged intensities of the molecular target of Onabot/A SNAP-25 (A; 25 kDa), the neuronal growth marker GAP43 (B; 43 kDa), and the markers of bladder sensory and parasympathetic cholinergic innervation, TRPV1 (C; 75 kDa) and VAChT (D; 60 kDa) obtained from bladder tissue of spinal intact and spinal cord injured rats 1 week and 4 weeks after lesion. cSNAP-25, cleaved form of synaptossome associated protein of 25 kDa; GAP43, growth-associated protein 43; INT, spinal intact; SCT, spinal cord transection; SNAP-25, synaptossome associated protein of 25 kDa; TRPV1, transient receptor potential vanilloid 1; VAChT, vesicular acetylcholine transporter. *p≤0.05 versus INT; #p≤0.05 versus SCT 1wk; One-way ANOVA followed by Tukey's multiple comparison test.

Effects of early treatment with BoNT/A in bladder reflex activity 4 weeks after SCI

Spinal intact rats, sham-operated, treated with saline presented a normal pattern of bladder reflex activity (**Table 1; Figure 2A, E-H**). Bladder-wall injections of BoNT/A did not significantly alter any urodynamic parameter evaluated in spinal intact rats 4 weeks after sham surgery (**Table 1; Figure 2B, E-H**).

	Sham + Saline	**Sham + Onabot/A**	**SCT + Saline**	**SCT + Onabot/A**
Frequency (contractions/ min)	0.31±0.10	0.63±0.47	0.88 ±0.24 *	0.92±0.42 *
Peak pressure (cm H_2O)	25.64±2.84	33.13±16.86	40.13±6.44 *	37.55±8.00
Basal pressure (cm H_2O)	2.89±3.24	10.25±4.11	9.75±3.67	21.06±8.53 *** #
Amplitude (cm H_2O)	22.75±3.12	22.88±12.75	30.86±4.14	16.49±6.83 ##

Table 1: Urodynamic parameters from sham-operated and SCT rats treated during spinal shock with saline or Onabot/A. *p≤0.05, ***p≤0.001 versus Sham + Saline; # p≤0.05, ##p≤0.01 versus SCT + Saline; One-way ANOVA followed by Tukey's multiple comparison test. Onabot/A, OnabotulinumtoxinA; SCT, spinal cord transection.

Rats submitted to SCT surgery and treated with saline developed the typical urodynamic pattern of NDO 4 weeks after lesion, characterized by high intravesical pressures (**p<0.05; Table 1; Figure 2C, F**) and increased frequency of voiding contractions (**p<0.05; Table 1; Figures 2C, H**).

Bladder injections with Onabot/A during spinal shock did not markedly affect bladder function in both spinal intact and SCT rats (Table 1; **Figures 2D, F, H**), although significant increase in basal bladder pressure was observed in SCT animals treated with Onabot/A in comparison with saline treatment (Table 1; **Figures 2D, E**).

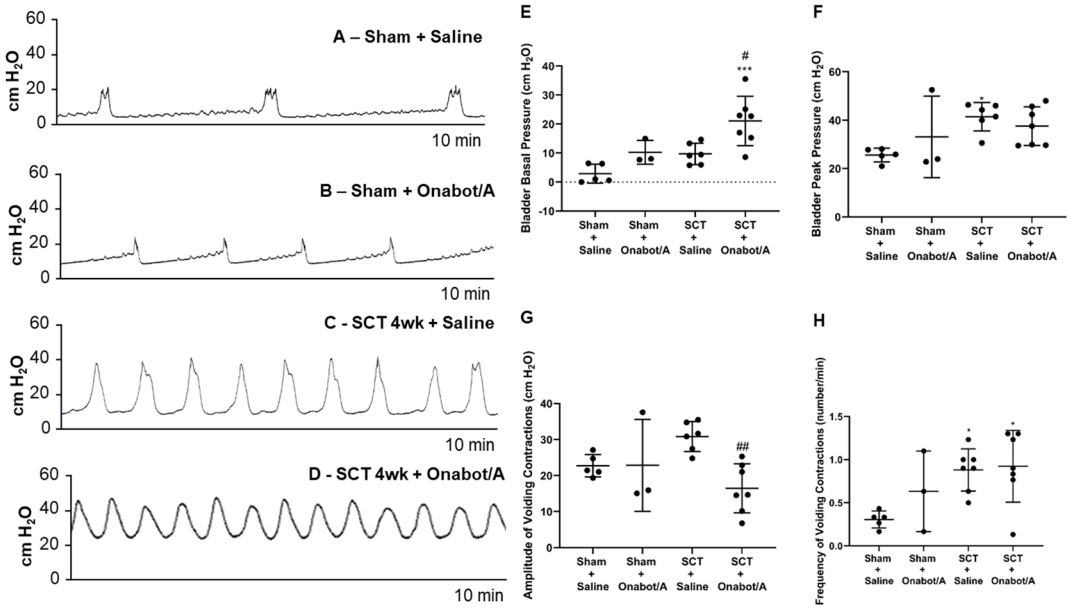

Figure 2: Representative cystometrograms obtained from spinal intact, sham-operated, rats treated with Saline (A) and Onabot/A (B) and 4 weeks after SCT rats treated with saline (C) and Onabot/A (D). Graphical representation of averaged basal (E) and peak (F) bladder pressures, amplitude (G) and frequency (H) of bladder contractions obtained for each experimental group. Graphs represent average values ± SD. *p≤0.05, ***p≤0.001 compared to Sham + Saline; # p≤0.05, ##p≤0.01 compared to SCT + Saline; One-way ANOVA followed by Tukey's multiple comparison test. Onabot/A, OnabotulinumtoxinA; SCT, spinal cord transection.

Effects of early treatment with BoNT/A in bladder tissue

To evaluate the effects of the neurotoxin activity in the bladder, bladder histological sections from saline- and BoNT/A-treated SCT rats were immunoreacted against the cleaved form of SNAP25 (cSNAP25). Little expression of cSNAP25 was found in toxin-treated animals (**Figure 3A**). Western blot analysis of the same tissues showed similar levels of SNAP-25, GAP43, TRPV1 and VAChT (**Figures 3B-E**).

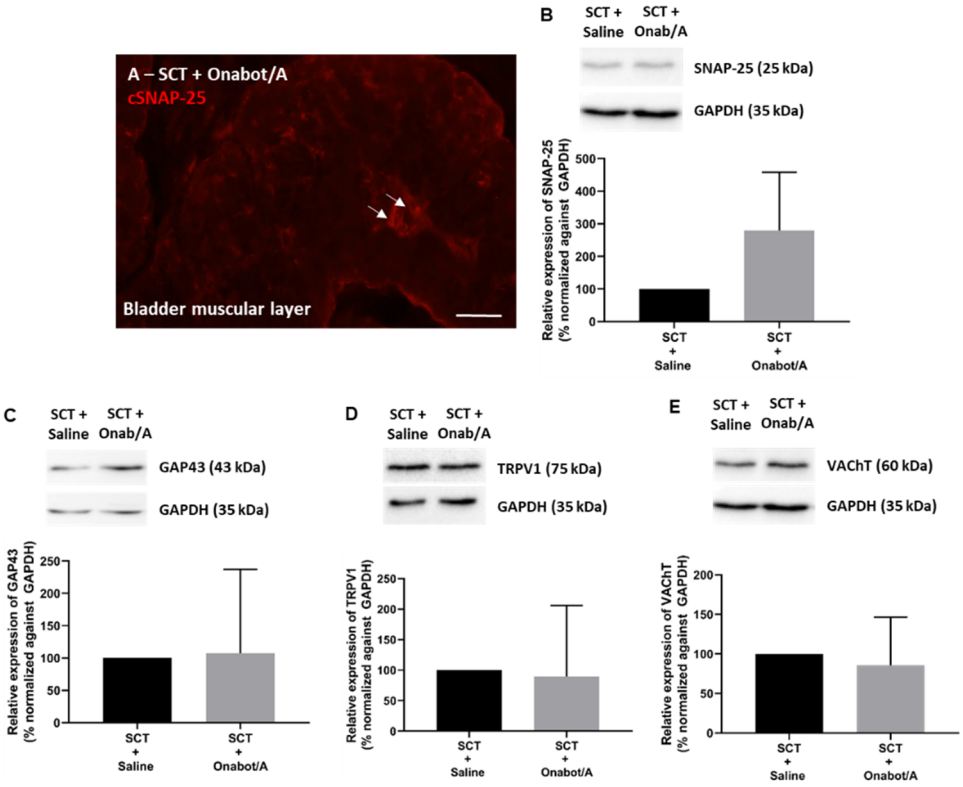

Figure 3: Effects of early intravesical administration of saline and Onabot/A in bladder innervation 4 weeks after SCT. (A) Representative image of a bladder section obtained from an SCT rat treated with Onabot/A showing immunoreactivity against the cleaved form of SNAP-25 (cSNAP-25). Scale bar represents 50µm. Representative Western immunoblotting bands and respective averaged intensities of the molecular target of Onabot/A SNAP-25 (B; 25 kDa), the neuronal growth marker GAP43 (C; 43 kDa), and the markers of bladder sensory, parasympathetic cholinergic and sympathetic adrenergic innervation, TRPV1 (D; 75 kDa) and VAChT (E; 60 kDa), all normalized against GAPDH (37 kDa). cSNAP-25, cleaved form of synaptossome associated protein of 25 kDa; GAP43, growth-associated protein 43; IR, immunoreactivity; Onabot/A and Onab/A, OnabotulinumtoxinA; SC, spinal cord; SCT, spinal cord transection; SNAP-25, synaptossome associated protein of 25 kDa; TRPV1, transient receptor potential vanilloid 1; VAChT, vesicular acetylcholine transporter.

Effects of early BoNT/A treatment in lumbosacral DRG and spinal cord

As in other studies [16], the expression of the neuronal stress marker ATF3 was investigated in DRG sections. The number of positive nuclei (**Figures 4A, B**) was similar in sections from SCT animals that had received either saline or BoNT/A.

In spinal cord sections from the same animals, the expression of the marker of neuronal activation c-Fos (**Figure 4C**), CGRP (**Figures 4D-F**) and GAP43 (**Figures 4G-I**) was also comparable between saline- and BoNT/A-treated SCT rats.

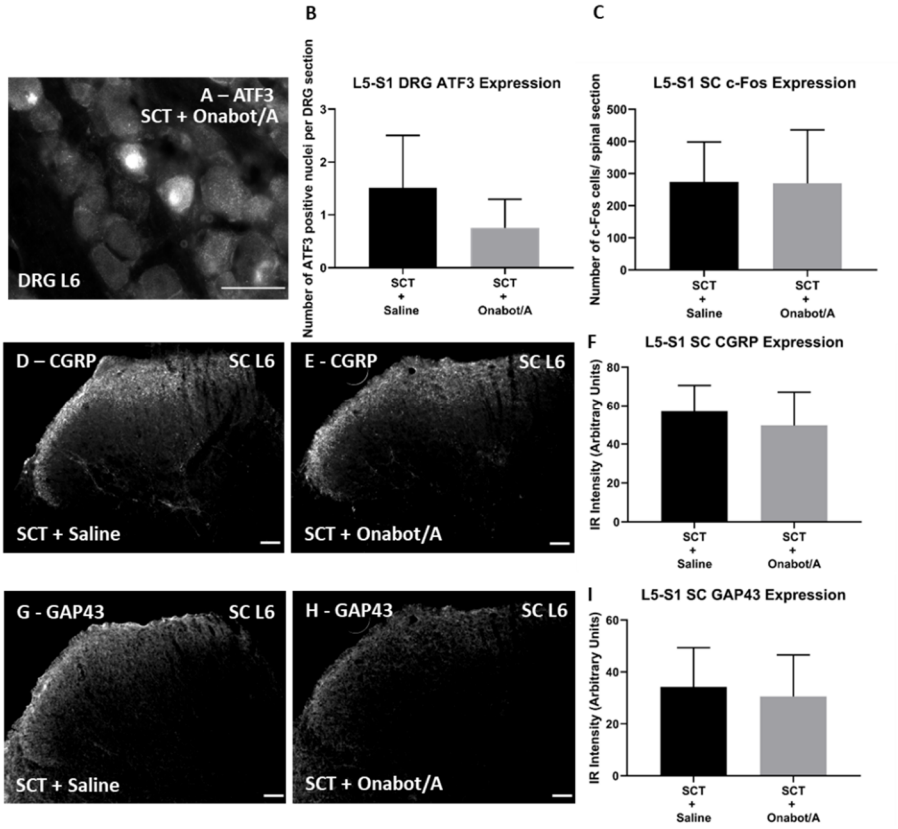

Figure 4: Effects of early intravesical administration of saline and Onabot/A in L5-S1 dorsal root ganglia (DRG) and spinal cord (SC) 4 weeks after SCT. (A) Representative image of an L6 DRG section from a SCT rat treated during spinal shock with Onabot/A showing immunoreactivity against ATF3 the transcription factor associated with neuronal stress in the nucleus of neurons. (B) Averaged number of ATF3 positive nuclei counted in DRG sections from SCT rats treated with saline or Onabot/A. (C) Averaged number of c-Fos expressing neurons counted in SC sections from SCT rats treated with saline or Onabot/A. Representative images of L6 spinal cord sections showing immunorecativity against CGRP (D, E) and GAP43 (G, H) in the superficial lamina of the dorsal horn, where terminal of bladder sensory afferents are located, and averaged relative intensities of immunostaining (F, I) obtained from saline and Onabot/A treated SCT rats. Scale bars represent 50µm. Graphs represent average values ± SD; t test. ATF3, activating transcription factor 3; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; GAP43, growth-associated protein 43; IR, immunoreactivity; Onabot/A, OnabotulinumtoxinA; SC, spinal cord; SCT, spinal cord transection.

Discussion

The vast majority of SCI patients develops NDO, after an initial period of bladder areflexia, termed spinal shock. Treatment is, in all cases, initiated with oral antimuscarinic drugs and for refractory patients, the gold standard therapy relies on BoNT/A injections in several points of the detrusor [5, 17]. Treatment is only initiated at chronic stages of disease, but recent studies indicate that intervening during the spinal shock, with protocols of neuromodulation or antimuscarinics, may prevent NDO establishment [11-13]. Here, we tested the hypothesis that early intradetrusor administration of BoNT/A could also block NDO development.

We used the rat model of large incomplete spinal transection at T8/T9, which causes NDO, easily identified in cystometries 4 weeks after spinal lesion [18]. We assessed the expression of SNAP-25, the target of BoNT/A, at different time-points after spinal lesion and found that spinal shock is accompanied by a significant decrease in bladder expression of SNAP-25. Our results suggest bladder denervation and impairment of neurotransmission in the bladder at 1 week after spinal lesion, as SNAP-25 is essential for neurotransmitter release [19]. Four weeks after SCI, SNAP-25 levels had increased and returned to control levels. GAP43 expression followed a similar patter, supporting remodelling of bladder innervation as NDO developed. Although non-significant, bladder TRPV1 also showed a trend towards downregulation at spinal shock and increased expression with NDO establishment. Bladder levels of VAChT also reduced 1 week after SCI and approximate to control levels 4 weeks post-SCI, being in agreement with previous observations [20].

The BoNT/A dosage (10 U per animal) was chosen according to a study by Behr-Roussel and co-workers who demonstrated long-lasting toxin effects in chronic SCI animals treated with 10 U of BoNT/A [14]. In contrast to clinical urology [21], in the present study the toxin was injected during spinal shock, when the bladder is areflexic or hypocontractile and neuroplastic

changes leading to NDO are ongoing. Cystometries were performed under urethane anaesthesia 4 weeks after surgeries and treatment. In comparison with sham animals, saline-treated SCT rats presented evident signs of NDO, including high frequency of bladder contractions, and increased intravesical pressure, both at baseline and at the peak of contraction. Early administration of saline or BoNT/A did not affect NDO but was devoid of secondary effects, as it also did not affect bladder reflex activity in sham animals. The only urodynamic parameter affected by intradetrusor BoNT/A was basal intravesical pressures in SCT rats. While an increase of basal intravesical pressure would be expected reflecting the use of urethane anaesthesia [22], such increase was only significant in BoNT/A-treated SCT rats. The reason for this can only be speculated but it could reflect a combined effect of early toxin delivery and urethane anaesthesia. Overall, these urodynamic observations indicate that early administration of BoNT/A had no effect on NDO emergence. Tissue analysis confirmed a lack of effect of the toxin. Thus, no differences were found in the expression of SNAP25, TRPV1 and VAChT. Likewise, GAP43 levels were also similar in bladder samples from sham and SCT animals treated with saline or BoNT/A in the spinal shock period. No changes were found in the number of ATF3-positive nuclei in lumbosacral DRG and spinal expression of CGRP, GAP43 and c-Fos.

As BoNT/A is the current gold-standard therapy for chronic NDO [23, 24], we hypothesized that early administration of the toxin would also beneficially impact on NDO development, in a similar fashion to what has been observed following early neuromodulation, oral anti-muscarinics and intravesical resiniferatoxin [12, 13, 25]. However, our results clearly point to a lack of toxin effect. The explanation for this could reflect the dynamics of NDO emergence. Indeed, one must not forget the mechanism of action of the toxin. BoNT/A is only internalized upon SV2 exposure during neurotransmitter release. The more active the neuronal terminals are, the more toxin is internalized, potentiating the intensity and extending the

duration of blockade of neurotransmitter release [17]. In the present study, the toxin was injected 3 days after spinal lesion. As in rodents the spinal shock lasts for 10 to 14 days [18], the time point chosen (3 days after spinal lesion) could have been too early to prevent NDO as the bladder is areflexic and nerve terminals coursing the bladder wall are most likely silent. This likely reflects loss of supraspinal input and absence of the developing lumbosacral spinal reflex that will lead to NDO. Therefore, it is possible that the injected toxin was not internalized and could nor exert its effects on bladder fibres. Non-internalized BoNT/A is very labile and quickly degraded by non-specific matrix metalloproteases [26]. This hypothesis could be tested in subsequent experiments in which bladder injections would take place later during the spinal shock.

In conclusion, although reports available in the literature have demonstrated that early therapeutic intervention could prevent NDO emergence, our results do not favour the use of BoNT/A for this purpose. However, additional experiments should be conducted at later time points of spinal shock, when some degree of bladder activity is already present and neuronal activity resumed in the bladder to fully concluded about the use of BoNT/A to harness NDO development.

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Publication III

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Effects of early intravesical administration of resiniferatoxin to spinal cord-injured rats in neurogenic detrusor overactivity

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Abstract

Objectives: To investigate if intravesical administration during spinal shock of resiniferatoxin (RTX), an ultrapotent desensitizing agonist of transient receptor potential vanilloid-1 (TRPV1), would silence TRPV1-expressing bladder afferents at an early stage of disease progression and modulate neurogenic detrusor overactivity (NDO) emergence.

Materials and Methods: Rats submitted to largely incomplete spinal cord transection at T8/9 spinal segment were treated with intravesical RTX (50 nM) or its vehicle during spinal shock. Four weeks after spinal lesion, bladder-reflex activity was evaluated by cystometry under urethane anesthesia, after which the bladder, spinal cord, and dorsal root ganglia were collected and processed.

Results: We found improvements on bladder function several weeks after early intravesical RTX administration, including a marked decrease of intravesical pressures and amplitude of bladder contractions. Such strong long-lasting urodynamic effects resulted from the very potent desensitizing activity of RTX on peripheral terminals of sensory afferents, an effect restricted to the bladder.

Conclusion: Our results support that an early intervention with RTX could potentially attenuate NDO development and ensuing urinary incontinence, with a dramatic impact on the quality of life of spinal cord injury patients.

KEYWORDS
afferent desensitization, bladder dysfunction, neurogenic detrusor overactivity, resiniferatoxin, spinal cord injury

Abbreviations: ATF3, activating transcription factor 3; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; FBS, fetal bovine serum; GAP43, growth-associated protein 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NDO, neurogenic detrusor overactivity; NGF, nerve growth factor; PB, phosphate buffer; PFA, paraformaldehyde; SCI, spinal cord injury; TRPV1, transient receptor potential vanilloid-1.

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1 | INTRODUCTION

It is well-established that supra-sacral spinal cord injury (SCI) is followed by the spinal shock period during which bladder is hypocontractile. Spinal shock is overcome by the emergence of a new reflex pathway, totally located at the lumbosacral spinal cord, resulting in neurogenic detrusor overactivity (NDO).[1] This condition is characterized by increased frequency of strong involuntary bladder contractions, often causing urinary incontinence episodes.

Treatment for NDO-induced urinary incontinence is well-defined and initiated by antimuscarinic drugs, with intradetrusor injections of botulinum toxin being the gold-standard treatment for refractory patients.[2] However, current interventions for urinary incontinence are only introduced once bladder dysfunction is a chronic condition, despite studies suggesting that initiating treatment at earlier time points could prevent bladder dysfunction and other SCI-induced problems. Indeed, it has been shown that pudendal nerve stimulation and sacral neuromodulation prevents NDO in dogs and humans[3,4] and early administration of fesoterodine to SCI rats improved bladder function several weeks after spinal lesion.[5] Together, these studies indicate that it is possible to control NDO emergence by early interventions in the initial stages of disease progression.

NDO is thought to result from massive neuroplastic changes occurring in the lumbosacral spinal cord after SCI, including sprouting of bladder afferents and enhanced activity of transient receptor potential vanilloid-1 (TRPV1)-expressing vesical afferents.[1] TRPV1 is abundantly expressed in sensory fibers coursing the bladder wall[6] and in urothelial cells.[7] Intravesical administration of resiniferatoxin (RTX), a potent TRPV1 agonist, induces desensitization of bladder C-fibers, even at very low concentrations, without producing significant noxious input.[8] In SCI patients, intravesical RTX significantly attenuated urinary incontinence and increased bladder capacity.[9] As in animals,[6] RTX-induced improvement of bladder function was accompanied by a marked reduction in bladder TRPV1 expression.[10] In the present study, we investigated if intravesical RTX administration during spinal shock in rats submitted to spinal cord transection would modulate NDO development.

2 | MATERIALS AND METHODS

2.1 | Animals and drugs

Experiments were performed in in-house bred, 220 to 290 g female Wistar rats, maintained under a 12-hour light/ dark schedule, with ad libitum access to food and water.

Spinal lesions or sham manipulations of the cord were performed under deep anesthesia induced by intraperitoneal injection of ketamine (60 mg/kg) and medetomidine (0.25 mg/kg). Intravesical administration of RTX (RTX; 50 nM; Sigma-Aldrich, Sintra, Portugal) or its vehicle (10% ethanol diluted in sterile saline) was performed under isoflurane anesthesia (4% for induction; 1.5% for maintenance). For cystometries and terminal handling, animals received a subcutaneous injection of urethane (1.2 g/kg).

Antibodies and reagents used in cell culture experiments are indicated in Table S1.

2.2 | Large incomplete spinal cord transection and treatments

The large incomplete spinal cord transection (SCT) at thoracic spinal segments T8/T9 was used as model of spinal injury. After anesthesia and dorsal shaving, the T13 vertebra was identified by palpation and the T7 to T10 vertebra exposed. Following a laminectomy, the spinal segments T8-T9 were visualized and a scalpel was inserted approximately 5 mm perpendicularly until the tip touched the bone. All surgeries were performed by the same experimenter to ensure reproducibility. The scalpel was removed and a small piece of sterile gelfoam was inserted to limit bleeding. Control animals underwent sham surgery and the spinal cords left intact (INT). All animals were left to recover, receiving daily ciprofloxacin (1 mg/kg) for 10 days after surgery. Bladders were manually emptied by abdominal compression twice a day for a period of 2 to 3 weeks.

Animals were divided into experimental groups and treated as described in Table 1. Solutions were slowly injected via a urethral catheter until the bladder reached its maximal capacity and left for 30 minutes. The concentration of RTX chosen was based in previous studies that demonstrated clinical efficacy of a 50 nM solution.[9,11]

TABLE 1 Experimental groups used in the present study, including the number of animals per group

Group	Surgical manipulation of the spinal cord	Intravesical treatment	Time of cystometry and tissue collection (weeks after surgery)
A (n = 4)	INT (sham surgery)	None	–
B (n = 4)	SCT 1 wk	None	1
C (n = 4)	SCT 4 wk	None	4
D (n = 12)	SCT 4 wk	Vehicle solution (10% ethanol in saline) at the surgery day (day 0; n = 4) or at 3 and 9 d after surgery (n = 8)	4
E (n = 12)	SCT 4 wk	RTX 50 nM at the surgery day (day 0; n = 4) or at 3 and 9 d after surgery (n = 8)	4
F (n = 4)	INT (sham surgery)	Vehicle 3 and 9 d after surgery	4
G (n = 4)	INT (sham surgery)	RTX 50 nM 3 and 9 d after surgery	4

Abbreviations: INT, intact; RTX, resiniferatoxin; SCT, spinal cord transection.
Note. Considering previous studies about the effects of intravesical RTX,[9] à priori analysis indicated that for a power of at least 0.8, the number of animals per experimental group should be 12. However, considering the severity of the protocols and that statistical significance was achieved with smaller number of animals per group, the number of animals used was as shown above.

2.3 | Cystometries

The bladder-reflex activity was evaluated under urethane anesthesia with constant body temperature. Following abdominal incision, a 21-gauge needle, connected to an infusion pump and a pressure transducer, was inserted into the bladder dome. Twenty minutes later, saline infusion was initiated (6 mL/h). The urethra remained unobstructed during cystometry and two animals from groups D and E were removed from analysis as due to urinary retention. Cystometric parameters were obtained from cystometrograms using LabScribe software (iWorx; World Precision Instruments, Hitchin, UK). After 1-hour-cystometry bladders of SCT rats treated with either vehicle or RTX, were manually emptied and the infusion rate of saline was increased to 10 mL/h to determine the intravesical volume necessary to induce urine release.

2.4 | Western blot analysis—bladder tissue

Bladders were collected immediately after cystometry and stored at −80°C until further processing. Samples were homogenized in lysis buffer. Seventy-five micrograms of protein was separated on 12% polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% milk in Tris-buffered saline containing 0.1% Tween-20 and incubated 48 hours at 4°C with primary antibodies, followed by incubation with specific horseradish peroxidase-conjugated antibodies. Bound antibodies were detected by chemiluminescence and digital images obtained using a Chemidoc MP System Imager and the ImageLab 5.1 software (Bio-Rad, Alfragide, Portugal). Membranes were reincubated (24 hours at 4°C) with an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody and signal detection repeated as above. Three samples per group were analysed and protocols were repeated three times. Levels of TRPV1, calcitonin gene-related peptide (CGRP) and growth-associated protein 43 (GAP43) were quantified by densitometry using the Fiji software.[12] The commercial anti-TRPV1 antibody used in the present study detected two bands, corresponding to splice variants of the receptor.[13] The two bands were measured and their intensity combined. All intensities measured were normalized against GAPDH.

2.5 | Perfusion and tissue analysis—spinal cord and dorsal root ganglia

After cystometry and collection of bladders, animals were perfused through the ascending aorta with calcium-free Tyrode's solution followed by 4% paraformaldehyde (PFA). Spinal L5-S1 segments and respective dorsal root ganglia (DRG) and the lesioned spinal cord (T8/T9) were collected, post-fixed for 5 hours in 4% PFA and cryoprotected overnight in 30% sucrose in phosphate buffer with 0.1% sodium azide. Transverse spinal cord (30-μm thick; L5-S1 and T8/T9) sections and longitudinal 12-μm thick DRG sections were obtained in a Leica cryostat (Leica, Famalicão, Portugal). Lumbosacral spinal sections were collected in a cryoprotective solution while DRG and T8/T9 spinal sections were collected in Superfrost Plus slides.

As before,[14] alternate sections from spinal cord and DRG were thawed, washed and blocked with 10% of normal horse serum (NHS) in phosphate-buffered saline Tween-20 (PBST) for 1 hour. The sections were then incubated for 48 hours at 4°C in primary antibodies. After several washes, sections

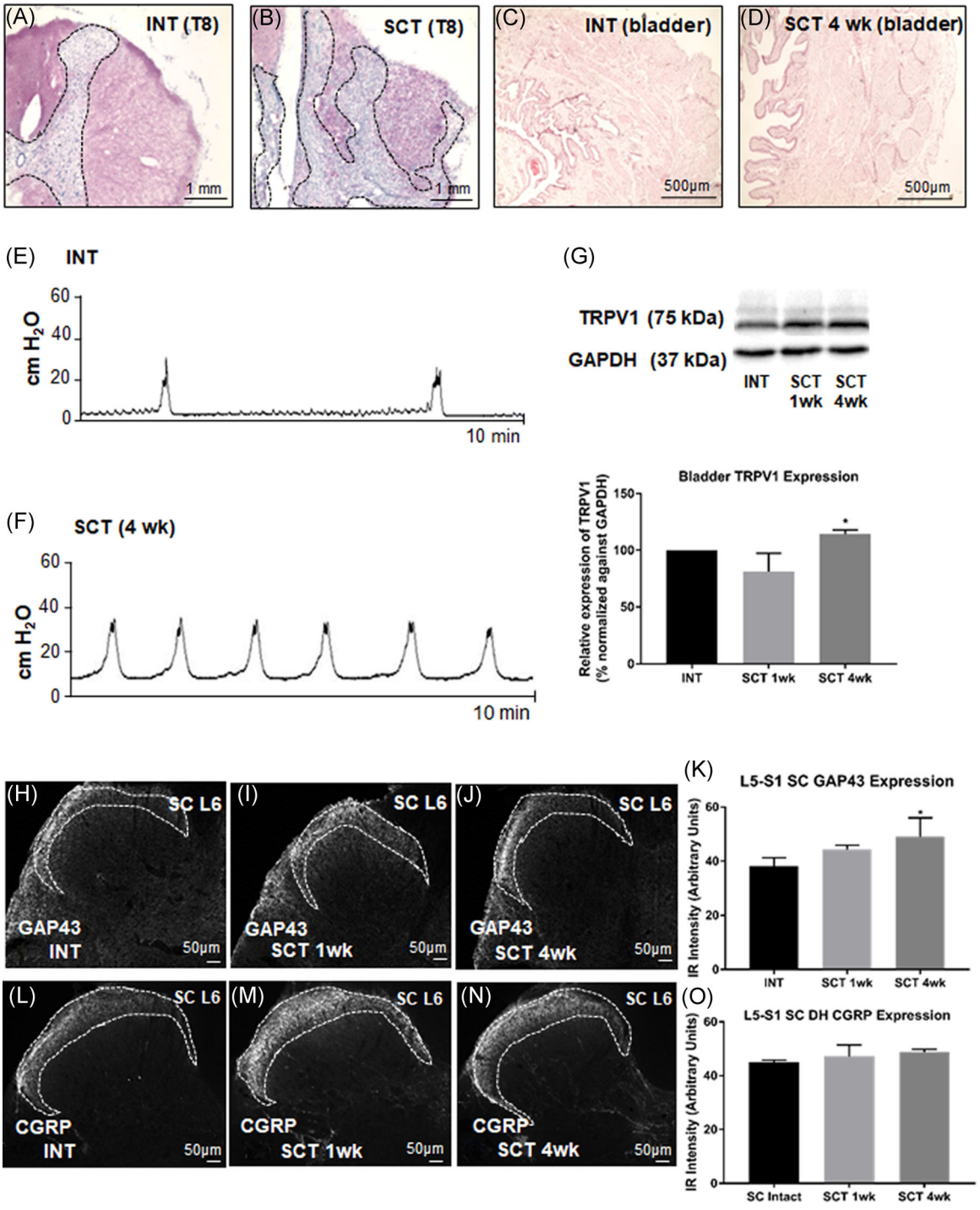

FIGURE 1 Time-dependent changes in bladder and spinal cord after spinal cord injury. Thionine-stained sections from T8 spinal cord of spinal intact (INT; A) and 4 weeks after spinal cord injury (SCT; B) rats show loss of integrity in white and gray matter as a consequence of a large incomplete cord transection. Histological H&E-stained bladder sections obtained from INT (C) and 4 weeks post SCT (D) rats evidence fibrosis and an increase in bladder wall thickness related to injury. Representative cystometric recordings from INT (E) and 4 weeks post SCT (F) rats show the development of the typical NDO pattern characterized by an increased number of bladder contractions and increased bladder pressures. Representative immunoblotting TRPV1 (75 kDa) and GAPDH (37 kDa) bands and averaged relative intensities obtained from bladders of INT, 1 and 4 weeks post SCT show an increase in bladder TRPV1 expression 4 weeks after SCT (G). Representative images show immunoreactivity (IR) against GAP43 (H-J) and CGRP (L-N) at the L6 spinal cord segment of INT, SCT 1-week, and SCT 4-week rats. Respective averaged IR intensities of GAP43 (K) and CGRP (O) evidence time-dependent sprouting of sensory afferents at the lumbosacral cord. Graphs represent average values ± SD. GAP43, growth-associated protein 43; CGRP, calcitonin gene-related peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin-eosin; NDO, neurogenic detrusor overactivity; SCT, spinal cord transection; TRPV1, transient receptor potential vanilloid-1. *$P \leq 0.05$; one-way ANOVA followed by the Tukey multiple comparison test

were incubated with species-specific Alexa flurochrome-labeled antibodies (Invitrogen - ThermiFisher Scientific, Porto, Portugal) for 1 hour at room temperature. DRG sections stained for activating transcription factor 3 (ATF3) were further incubated overnight in biotinylated IB4, followed by PBST washes and 1-hour incubation in fluorochrome 488-conjugated streptavidin. The sections were mounted with Prolong Gold mounting medium (Molecular Probes, Porto, Portugal) and representative images were obtained in a Zeiss microscope (Axioimager Z1; Zeiss, Berlin, Germany) using the Axiovision 4.8 software. Spinal CGRP and GAP43 immunostaining was quantified by densitometry using the Fiji software.[12] Intensity was averaged from 15 to 20 spinal sections per animal by selecting the dorsal horn area exhibiting immunoreactivity (laminae I-IV), and deducting background intensity. In DRG, the number of nuclei positively labelled for ATF3 was averaged after counting (25-30 sections per animal). Spinal T8/T9 cord sections were stained using the thionine protocol to analyze lesion extension.

Bladders obtained from INT and 4-week-SCT animals were washed in PBS at 4°C for 7 hours and fixed in 10% buffered formalin for 24 hours. After paraffin embedding, bladders were sectioned at 5 mm and stained with hematoxylin-eosin to analyze tissue histology.

2.6 | In vitro studies: DRG cell culture

After cystometry, L5-S1 DRG were collected from vehicle and RTX-treated SCT animals (n = 3 per group) and placed in Dulbecco's modified Eagle's medium-F12 (10% fetal bovine serum [FBS] and 1% penicillin/streptomycin). Cell culture was done as in other studies[14] and included a digestion with 0.125% collagenase (2 hours at 37°C), tissue dissociation, purification, and duplicate cell plating onto poly-L-lysine and laminin-coated coverslips, maintained at 37°C in a humidified 5% CO_2 atmosphere for 12 hours. This was followed by fixation with ice-cold 4% PFA for 15 minutes, several washes and incubation for 5 minutes in 0.1% of sodium borohydride. After PBS washes and 1-hour blocking with 5% FBS in 0.4% PBS-Tween-20, cells were incubated in anti-β3-tubulin antibody, with subsequent washes and further incubation with a species-specific Alexa Fluorochrome-labeled antibody. Coverslips were mounted with Vectashield medium (Vector Laboratories, Peterborough, UK) and images collected in an Axioskop 40 microscope with the AxioVision 4.6 Software (Carl Zeiss) for quantification of neurite branching and total neurite length analysis with SYnapse Detector (SynD).

2.7 | Statistical analysis

Statistical analyses were performed in GraphPad Prism 6 Software (GraphPad, San Diego, CA) using a *t* test or one-way repeated-measure analysis of variance; followed by the Tukey multiple comparison test. All values are presented as mean ± SD and $P < 0.05$ was considered statistically significant. All intergroup comparisons were evaluated and are indicated.

3 | RESULTS

3.1 | Largely incomplete spinal cord transection alters bladder function and histology

Histological analysis of the spinal T8/T9 segments and bladders obtained from groups A-C showed evident

TABLE 2 Cystometric findings from intact and SCT rats after early treatment with vehicle and RTX

	Frequency of voiding contractions (no. of contractions/min)	Frequency of voiding contractions (no. of contractions/min)	Baseline pressure of voiding contractions, cm H_2O	Peak pressure of voiding contractions, cm H_2O	Amplitude of voiding contractions, cm H_2O
Intact + vehicle	0.37 ± 0.13	0.21 ± 0.02	3.50 ± 2.80	28.01 ± 3.96	24.51 ± 3.44
Intact + RTX	0.54 ± 0.05	0.02 ± 0.02	18.17 ± 5.21 $^{****}P < 0.0001$	37.40 ± 4.54	19.23 ± 6.31
SCT + vehicle	0.71 ± 0.20*** $^{***}P = 0.0006$	0.44 ± 0.14 $^{**}P = 0.0100$	11.59 ± 5.37 $^{**}P = 0.0012$ $^{\$}P = 0.0456$	42.44 ± 9.81 $^{***}P = 0.0006$	34.03 ± 9.83 $^{*}P = 0.0223$ $^{\$\$}P = 0.0032$
SCT + RTX	0.46 ± 0.16 $^{\#\#}P = 0.0086$	0.11 ± 0.07 $^{\#\#\#\#}P < 0.0001$	14.00 ± 2.48 $^{****}P < 0.0001$	31.93 ± 5.28 $^{\#\#}P = 0.0088$	18.89 ± 2.76 $^{\#\#\#}P = 0.0001$

Abbreviations: RTX, resiniferatoxin; SCT, spinal cord transection.

Note. Data presented as mean ± standard deviation.

$^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, and $^{****}P \le 0.0001$, compared to Intact + Vehicle group.

$^{\#\#}P \le 0.01$ and $^{\#\#\#}P \le 0.001$, compared to SCT + Vehicle group.

$^{\$}P \le 0.05$ and $^{\$\$}P \le 0.01$, compared to Intact + RTX group.

changes after spinal lesion. Four weeks post lesion there was an almost entire loss of gray-matter arrangement in dorsal and ventral horns, with white-matter disruption and several cavities (Figure 1B) absent in sections from spinal intact (Figure 1A). These histological changes are consistent with a large incomplete spinal transection as spared tissue was present in most sections obtained from the lesioned area, albeit in very small quantities at some places. At the same time points, histological changes were also present in bladder sections with a significant increase in bladder wall thickness (629.7 ± 331.1 μm in spinal intact animals and 1236 ± 117.4 μm at 4 weeks post SCI; $P = 0.021$) (Figure 1C,D).

Spinal T8/T9 injury induced marked changes in bladder function, with increased frequency of bladder contractions and higher peak pressures (Figure 1E,F). These changes were accompanied by a significant time-dependent increase in bladder TRPV1 and lumbosacral spinal GAP43 and CGRP expression (Figure 1 G-O; Table S2; $P < 0.05$ vs spinal intact).

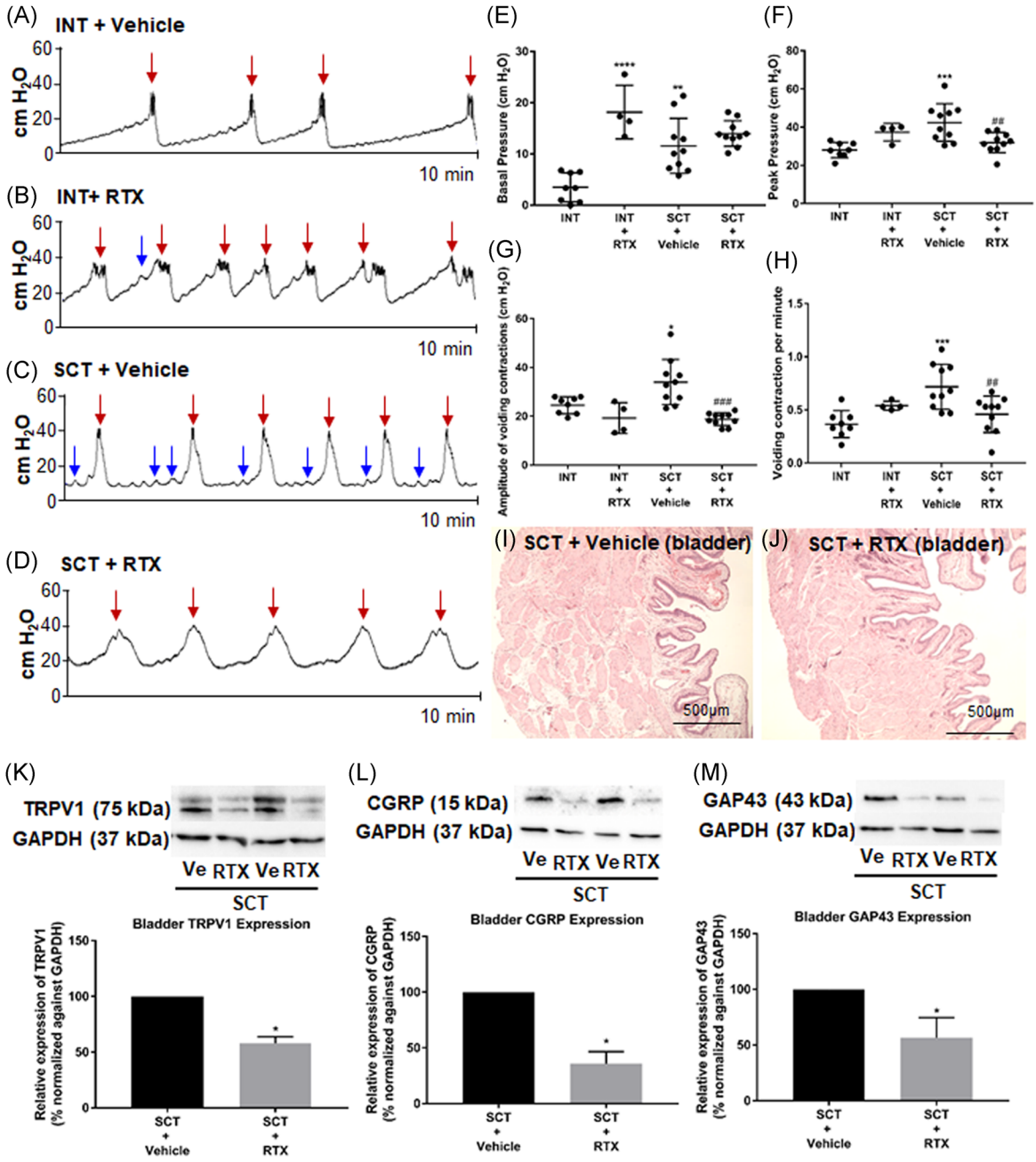

FIGURE 2 Continued.

3.2 | Early RTX administration improves bladder function in chronic SCT animals

All animals were daily evaluated after spinal lesion and submitted to abdominal compression for urine removal. There were no changes in the duration of the spinal shock period that could be attributed to intravesical treatment.

Vehicle-treated INT animals presented normal bladder-reflex activity without any major change in any cystometric parameter (Table 2; Figure 2A,E-H). INT rats that received intravesical RTX presented a significant increase in basal pressure ($P < 0.001$; Table 2; Figure 2B,E-H).

Four weeks after SCT, vehicle-treated rats presented urodynamic patterns indicative of NDO and similar to nontreated SCT animals (Table 2 and Figure 2C,E-H), with significant increases in all parameters analysed. Early intravesical RTX administration significantly improved bladder activity in SCT rats, irrespective of the day of intravesical treatment or the number of RTX instillations (Table 2 and Figure 2D,E-H).

Changes in volume of infused saline necessary to induce an expulsive bladder contraction were also found following early RTX. In SCT animals treated with early vehicle the infused volume necessary to induce urine release was 0.94 ± 0.034 mL and voiding occurred 5.66 ± 2.07 minutes after the beginning of accelerated saline infusion. In SCT rats treated with early RTX those values changed to 3.30 ± 0.60 mL ($P = 0.0018$ vs vehicle) and 20.23 ± 3.38 minutes ($P = 0.0013$), respectively.

3.3 | Early RTX administration after spinal cord injury reduces bladder TRPV1, CGRP, and GAP43 expression without changing histology

Four weeks after SCT and early RTX administration, TRPV1 expression in the urinary bladder had decreased by 42% (Figure 2K; $P = 0.0142$ compared to vehicle-treated animals). In contrast, gross bladder histology was not altered by the treatment (Figure 2I,J). The reduction in TRPV1 expression was accompanied by a significant reduction in the CGRP and GAP43 content (respectively, 64% and 44% decrease compared to SCT vehicle-treated animals; $P = 0.0293$ and $P = 0.0441$; Figure 2L,M).

3.4 | Early intravesical administration of RTX does not induce neuronal injury in lumbosacral DRG neurons

As RTX-induced reduction of bladder GAP43, TRPV1, and CGRP expression could reflect long-term injury to bladder afferents, the expression of the well-established marker of neuronal stress ATF3[15] was resolved by immunohistochemistry in sections from L5-S1 DRG, where perikarya of bladder afferents are located. Expression of ATF3 was found in the nuclei of a small number of DRG neurons, which lacked CGRP expression and did not bind IB4 (Figure 3A-C). No differences were found between the number of ATF3-positive nuclei between vehicle-treated (1.21 ± 0.57) and RXT-treated SCT rats (1.84 ± 1.19; Figure 3D).

Effects of early RTX on the intrinsic growth ability of DRG neurons, a measure of neuronal viability, were also

FIGURE 2 Effects of early intravesical administration of vehicle and RTX in bladder function, morphology and innervation 4 weeks after SCT. Representative cystometric recordings from spinal intact (INT) and 4 weeks SCT animals treated with early vehicle (A and C, respectively) and RTX (B and D, respectively) show that the typical pattern of NDO is present in vehicle-treated 4-week SCT rats but absent in SCT animals that received early RTX. Red arrows indicate representative voiding contractions and blue arrows show representative nonvoiding contractions. The amplitude of bladder contractions was calculated by subtracting minimal to maximal pressure values. Frequency was calculated as the number of bladder contractions with amplitude values greater than 10 cm H_2O occurring in a period of 30 minutes.[14] Graphics show average basal (E) and peak (F) pressures, amplitude (G) and frequency (H) of bladder contractions from all experimental groups and demonstrate a significant decrease in peak pressure, resulting in a marked decline of the amplitude of bladder contractions, and a reduction in frequency of voiding contractions following early RTX. Histological H&E-stained bladder sections obtained from 4-week SCT rats treated with vehicle (I) and RTX (J) evidence no alterations in bladder morphology related to treatment. Representative immunoblotting bands corresponding to TRPV1 (75 kDa) and GAPDH (37 kDa) in the bladder of 4-week SCT animals treated with vehicle (Ve) and RTX and averaged relative intensities (K) show a significant decrease in bladder TRPV1, the receptor for RTX, after early treatment with the toxin. Likewise, CGRP (15 kDa; L) and GAP43 (43 kDa; M) immunoblotting bands and averaged immunoreactivity intensities indicate a strong decrease in sprouting of peptidergic sensory bladder afferents following early treatment with RTX. Graphs represent average values ± SD. ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; GAP43, growth-associated protein 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin-eosin; NDO, neurogenic detrusor overactivity; RTX, resiniferatoxin; SCT, spinal cord transection; TRPV1, transient receptor potential vanilloid-1. In graphs E-H $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared to INT and $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared to SCT + Vehicle; one-way ANOVA, followed by the Tukey multiple comparison test. In graphs K-M, $^{*}P \leq 0.05$; *t* test

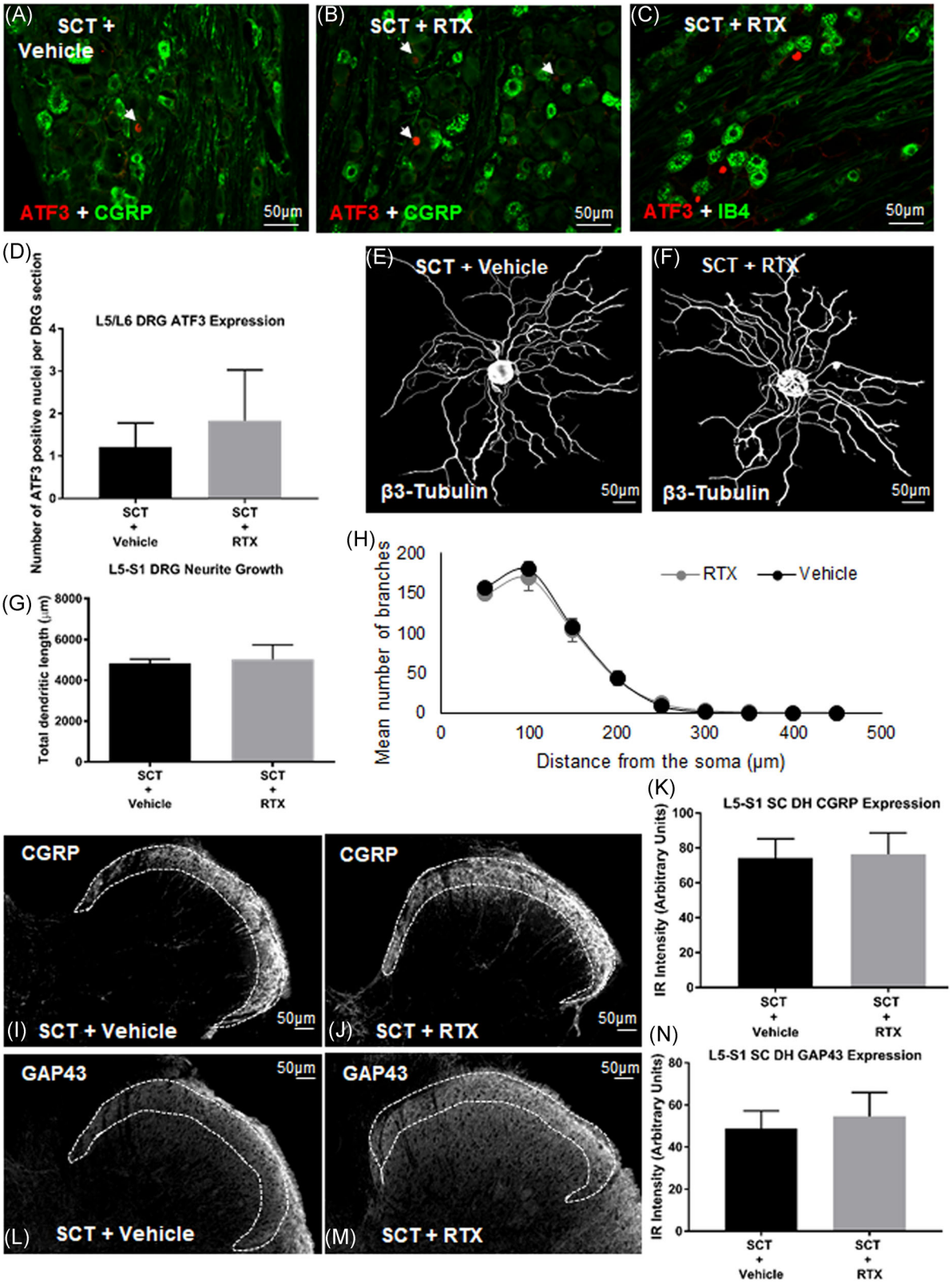

FIGURE 3 Continued.

evaluated in in vitro conditions with abundant concentration of nerve growth factor (NGF) in the medium. All L5-S1 DRG neurons collected from vehicle- or RTX-treated SCT animals attached to the substrate and emitted long neurites (Figure 3 E,F). No differences were found between groups in the average length of neurites (5.01 ± 0.72 mm and 4.83 ± 0.21 mm, respectively in vehicle- and RTX-receiving rats) or neurite branching (Figure 3G,H).

3.5 | Early intravesical RTX administration does not alter spinal expression of CGRP and GAP43

To investigate the effects of early RTX at the lumbosacral (L5-S1) spinal cord, where the majority of bladder afferents central projections terminate, spinal expression of CGRP and GAP43 was resolved. Intense CGRP immunolabeling in the superficial laminae of the cord was observed (Figure 3I,J), with some processes present in deeper laminae. GAP43 was also found in laminae I-II (Figure 3L,M) but not in deeper areas of the cord. In both cases early RTX did not change spinal expression of these proteins (Figure 3 K,N).

4 | DISCUSSION

Management of SCI-induced urinary incontinence has always been focused on dealing with a chronic condition rather than attempting to harness its development.[16] Recent studies, however, suggest that early interventions are of clear benefit for SCI patients whether they aim to reduce pain and spasticity[17,18] or improve bladder function.[3,4] Accordingly, here we found that early intravesical RTX administration significantly attenuated NDO development, acting in peripheral structures without effects on spinal afferent terminations.

In the present study, we used a rodent model of largely incomplete spinal transection. The concentration of RTX used (50 nM) had already been shown to be effective in reducing bladder hyperactivity.[9,11] Administration of this neurotoxin was performed during spinal shock, when the bladder is areflexic or hypocontractile and it is assumed that neuroplastic changes leading to NDO are occurring.[1] Four weeks after treatment and irrespective of the time of intravesical treatment or the number of exposures to RTX, improvement of bladder function was evident and still present 8 weeks after treatment (data not shown), in agreement with other studies.[19] This is an important outcome which indicates that early intravesical RTX administration may mitigate the development of high intravesical pressures in a long-lasting manner, an important matter in NDO management.[20] Importantly, we found an increase in basal intravesical pressures after RTX treatment, suggesting urinary retention, which, in patients could concur to reduce urinary incontinence, provided patients are following a clean intermittent catheterization regime for timely urine removal. However, it should be recalled that this this observation could merely reflect the effects of urethane,[21] as cystometries were performed under anesthesia.

As in SCI patients,[22] bladder TRPV1 was upregulated following largely incomplete spinal transection. TRPV1 overexpression was significantly reduced after early intravesical RTX, despite treatment having taken place 4 weeks earlier. These observations agree with previous studies demonstrating decreased TRPV1 bladder levels as early as 24 hours posttreatment[6] and indicate that RTX effects on the expression of its receptor are long-lasting.[19] In the urinary bladder, TRPV1 strongly colocalizes with CGRP.[6] Accordingly, CGRP levels were also reduced following early intravesical RTX treatment, confirming the effect this neurotoxin on peptidergic fibers.[6]

Like TRPV1 and CGRP, the expression of axonal growth GAP43 was decreased in the bladder following early RTX. It is likely that improvement of bladder function by early RTX could have resulted from blocking axonal expansion of peptidergic terminals in the bladder wall, possibly reflecting decreased NGF content in the bladder wall.[23] The reasons for this could reside in the well-known effect of vanilloids on axonal transport.[24] Our cell culture experiments suggest

FIGURE 3 Effects of early intravesical administration of vehicle and RTX in lumbosacral DRG and spinal cord 4 weeks after SCT. Representative images show immunofluorescence staining for the neuronal stress marker ATF3 (red) and CGRP or IB4 (green) in the lumbosacral DRG from SCT rats treated with vehicle (A) and RTX (B, C). ATF3 expression is restricted to the nuclei of cells and no positive nuclei is found in CGRP-immunoreactive and IB4-binding perikarya. No differences were found between treatments, suggesting that neurons did not suffer any injury related with intravesical treatment (D). When cultured in controlled conditions with abundant NGF, DRG neurons collected from SCT animals receiving early vehicle (E) or RTX (F) show a similar pattern of neurites extending (G) and ramification (H) irrespective to treatment, as shown by anti-β3-tubulin immunostaining. Representative images show a similar pattern of CGRP (I,J) and GAP43 (L, M) immunoreactivity in the superficial lamina of dorsal horns of L6 spinal cord segments obtained from vehicle-RTX-treated rats. No changes on immunoreactivity intensities of CGRP (K) and GAP43 (N) were found after early administration of RTX. Graphs represent average values ± SD; *t* test. ATF3, activating transcription factor 3; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; GAP43, growth-associated protein 43; NGF, nerve growth factor; RTX, resiniferatoxin; SCT, spinal cord transection

that NGF deprivation due to impaired transport might indeed be involved in reduced axonal sprouting following early RTX as DRG neurons collected from vehicle- and RTX-treated SCT animals presented similar patterns of axonal branching when cultured in controlled conditions with abundant NGF.

As the effects on bladder function were evident at 4 weeks after RTX, we hypothesized that the toxin might have induced damage to DRG neurons. To investigate this, we evaluated the expression of ATF3, an established marker of neuronal response to peripheral injury.[15] We found ATF3-positive nuclei in sections from both vehicle and RTX-treated SCT animals. ATF3 levels were similar between groups, indicating that the putative neurotoxic effects were not reflected in ATF3 expression. Although this might suggest that RTX did not induce a severe injury to DRG neurons, 4 weeks might be enough time to resolve whichever neuronal damage might have been induced by RTX. In fact, it should be noted that at the same time point following RTX instillation the expression of the injury markers galanin and c-Jun in DRG neurons had already returned to control values.[25]

In contrast to the bladder, no changes were seen in CGRP and GAP43 levels at the lumbosacral spinal cord. The reasons for this may only be speculated but it is possible that intravesical RTX could have blocked trafficking of CGRP and GAP43 from the cell bodies to the bladder, without affecting trafficking to the central processes terminating in the dorsal horns. This is in line with studies demonstrating a reduction in the expression of bladder CGRP following peripheral colchicine treatment without changes in the lumbosacral spinal CGRP expression.[26]

Altogether, results presented support the benefits of early intravesical administration of RTX to attenuate NDO development. Our data adds to the growing body of evidence[3-5] that postulates that the anticipation of therapeutic intervention might avoid degradation of bladder function, with a clear positive impact on the SCT patients' quality of life.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

Experimental procedures were carried out according to the European Communities Council Directive 2010/63/EU and approved by local authorities.

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Final Considerations

1. Improving treatment of NDO: the use of BoNT/A

Since the first application of BoNT/A in clinical urology [1], the toxin has been extensively studied being currently recognized as an effective and powerful therapeutic tool. It is an established and licensed therapy for neurogenic detrusor overactivity (NDO) and idiopathic overactive bladder (OAB) and it is also used off-license in the treatment of bladder pain syndrome (BPS), DSD and benign prostatic hyperplasia (BPH) [2, 3]. Improvements in urinary function and reduction of pain levels reflect toxin-mediated blockade of neurotransmission, affecting both efferent and afferent neuronal pathways that control LUT function [4, 5]. BoNT/A wide-ranging, long-lasting but reversible effects on LUT innervation, adding to its safe administration and rare secondary effects support its wide use in clinical urology [6].

1.1. Conversion of Onabot/A and Abobot/A for bladder wall injections (Publication I)

Multiple toxin formulations are currently commercially available from different vendors [7], although Onabot/A has been the most well-studied and most commonly used in clinics [8]. Onabot/A is the only formulation of botulinum toxin A approved by competent authorities for management of specific LUT pathologies, with dosages of 100 U and 200 U indicated for OAB and NDO [5]. Abobot/A is also frequently used in clinical urology, albeit in off-label conditions. This formulation is currently in phase 3 clinical trials for the management of neurogenic bladder [9].

All formulations of BoNT/A include the 150 kDa neurotoxin domain, responsible for the catalytic effect on SNAP-25 but the amount of the active toxin in Onabot/A and Abobot/A commercial vials differ. While flasks of Onabot/A contain 0.9 ng, the Abobot/A containers hold 2.69 ng [10]. This difference results from the presence of structural accessory proteins, necessary to stabilize the toxin. Thus, the molecular weight of Onabot/A is 900 kDa and of Abobot/A is 500 kDa [11]. The recommended dosages for each formulation are defined with bioessays, but companies have used different analytical methods. Therefore, it is difficult to

determine an equivalence between unitary doses of toxin formulations [11]. Although Onabot/A potency is determined by the EC50 (amount of toxin required to provoke a response halfway between the baseline and the maximum response) of cSNAP-25 in differentiated neuroblastoma cells, Abobot/A potency is determined by the mouse LD50 (amount of toxin that kills 50% of the mice) [12-14]. Because it may be important in the clinical setting to choose between Onabot/A and Abobot/A, some studies compared the effects of unitary doses of toxins formulations in skeletal muscle [8, 15, 16]. In the bladder, some works suggested dose equivalence ratios varying between 1:3 to 1:5 Units of Onabot/A and Abobot/A, but results were not thoroughly confirmed and often challenged by other investigators [17, 18].

The first publication included in this dissertation (**Publication I**) addresses this issue and aimed to accurately identify a dose conversion ratio for Onabot/A and Abobot/A for bladder smooth muscle injections. Our experimental procedure consisted of injecting the same amount of Onabot/A and Abobot/A, diluted in equal saline volume, in a single apical point of the mouse bladder dome. The relative potency for each formulation was evaluated based on its catalytic capacity and the number of bladder fibres expressing the cleaved form of SNAP-25 was counted. The main finding of our study was that Onabot/A is 1.6 times more efficient than Abobot/A, when targeting bladder nerve fibres and cleaving SNAP-25. As Onabot/A is commercially available in vials of 50 U and 100 U while Abobot/A flasks contain 500 U, the results presented in **Publication I** indicate that the Onabot/A formulation might be easier to use and to adjust the amounts needed, in order to avoid overdosing. In fact, the use of excessive doses of Abobot/A in clinic urology, when converting amounts between the two formulations, might underlie the reported higher rates of severe urinary retention in comparison to Onabot/A [17]. Overdosing should be avoided to minimize kidney damage due to urinary retention and to decrease probabilities of developing antibodies against BoNT/A that compromise treatment efficacy [19].

In this study, the diffusion pattern was also observed as spread of the injected volume could reflect on the effects of the toxin on bladder contractibility. Studies from other groups

suggested limited spread from the site of injection, exclusively dependent on the volume injected [15, 20]. Thus, dilution of BoNT/A products should be considered based on the area of the muscle to treat [21]. Here, the dose of 0.5 U of toxin formulations was diluted in a total volume of 2 µL of saline, which was chosen based on toxin ability to cleave SNAP-25. However, irrespective of the BoNT/A formulation, the number of cSNAP-25 expressing fibres was variable among experimental animals injected. We propose that the fluctuations observed are due to inevitable errors associated with one unique injection in a particularly thin bladder wall of mice. As these fluctuations also reflect the experimenter's dexterity, this should be considered in clinical practice, where the procedure often consists in 20 to 30 bladder injections [22].

Importantly, accurate therapeutic dosages of each one of the formulations should be further evaluated in the scope of different pathological conditions in experimental animals and human patients, once physical spread, passive diffusion and migration through axonal or haematogenous transport cannot be disregarded as contributors to toxin's efficacy and adversities [23].

1.2. Effect of intrathecal BoNT/A in chronic NDO as an alternative for bladder injections (Publication II)

Pioneer works from Schurch and co-worker demonstrated that intradetrusor injection of Onabot/A improved bladder function and abolished urinary urgency in neurogenic patients [24, 25]. Subsequent studies confirmed and expanded these initial observations and in 2011 bladder injections of Onabot/A were licensed by the FDA for the treatment of NDO [26-29]. Today, intradetrusor Onabot/A is the gold standard option for patients that are refractory to oral medication [5]. Treatment efficacy relies on the toxin ability to block neurotransmission in bladder nerve fibres. Urodynamic evaluation of patients following treatment clearly shows long-lasting improved bladder function, with a marked reduction in bladder contractions and in the number of UI episodes, still present 6 to 9 months after treatment [26, 27, 30].

BoNT/A is a non-specific neurotoxin and targets the different nerve fibres coursing in the bladder wall. The high affinity toxin receptor SV2 is present in 95% of parasympathetic, 69% of sympathetic and 58% of sensory bladder fibres [31, 32]. Following bladder injection, the cleaved form of SNAP-25, a marker of the proteolytic activity of the neurotoxin, was present in all fibres [33], but predominantly in cholinergic and sensory fibres in detrusor and suburothelial bladder layers [31]. Therefore, reduction in urgency and bladder contractions after BoNT/A delivery most likely reflects the effect of the neurotoxin in blocking release of acetylcholine from cholinergic nerve terminals and activation of muscarinic receptors in smooth muscle cells [34]. Because urothelial cells are also able to release acetylcholine [35], this does not exclude an effect on the urothelium [36, 37]. However, excessive inhibition of parasympathetic neurotransmission could result in urinary retention, a problem referred by some patients [28].

The contribution of efferent neuronal pathways to the establishment of NDO seems to be limited and the leading role is played by afferent C-fibres, which undergo sprouting at the lumbosacral spinal cord [38]. This results in massive spinal synaptic rearrangement and, ultimately, in a new spinal circuit, responsible for abnormal bladder function. Therefore, one can hypothesize that targeting bladder C-type afferents at the spinal cord level could be beneficial and reduce urinary dysfunction after SCI. This is supported by a study demonstrating that intrathecal administration of BoNT/A improved bladder reflex activity and reduced pain in an animal model of chronic cystitis by specifically targeting peptidergic afferents [39]. Therefore, in **Publication II** we explored the intrathecal route for BoNT/A administration as a way of specifically blocking bladder sensory afferents in animals SCI-induced NDO and assessed the effects on bladder reflex activity.

The rat model of thoracic spinal cord complete sectioning was used and animals presented evident signs of NDO 4 weeks after lesion (increased intravesical pressures and frequency of bladder contractions) [38, 40]. Such urodynamic changes were accompanied by sprouting of peptidergic sensory afferents at the L5-L6 lumbosacral spinal cord, and increased

expression of calcitonin gene related peptide (CGRP), as also observed by others [41]. As before, [39], 5 units of BoNT/A, diluted in 50 µL of saline, were injected in the intrathecal space of 4-week SCI animals. This resulted in improved bladder reflex activity, with significant decrease in voiding frequency and bladder basal pressures. This was accompanied by marked cleavage of SNAP-25 mainly in laminae I and II of the spinal L5-6 and a significant reduction in CGRP expression at lumbosacral spinal cord and DRG in response to IT Onabot/A, suggesting an effect of the toxin on peptidergic sensory afferents.

While BoNT/A effects on bladder efferent nerves have been widely studied and are particularly well described, effects on sensory afferents are less well understood [42]. Together with previous studies [39], these observations indicate that BoNT/A-mediated improvement of bladder function in pathological conditions may result from the effects of the toxin on sensory fibres. Accordingly, ATF3, a marker of cellular injury, was found in the nuclei of DRG neurons. CGRP expression in the soma of DRG neurons was also affected. Together, these results suggest that BoNT/A-mediated improvement of bladder function in pathological conditions may also results from an effect on sensory fibres without affecting efferent nerves and causing urinary retention. In the future, NDO management might be improved by restricting the action of this toxin to sensory afferents, possibly by producing recombinant botulinum toxins.

2. Can NDO be prevented?

NDO is very common amongst SCI patients, affecting approximately 90% of individuals with high thoracic lesions [43-45]. Initially, during spinal shock, management of urinary dysfunction aims to guarantee bladder emptying. When this is overcome and NDO has emerged, additional treatments initiate with the goal of reducing the frequency and/or intensity of bladder contractions and, if present, control DSD [46, 47]. This results in control of urinary incontinence and intravesical pressures. Of note, treatment is only initiated when NDO is a chronic, established condition, with little chances of reverting to a more favourable situation.

While the current approach consists in waiting for the disorder to develop and then initiate treatment, a new trend has been attracting increasing attention. Because prevention is always better than cure, some studies have sought to investigate if NDO emergence can be blocked. Sievert and co-workers were pioneers in the field, initiating sacral neuromodulation during spinal shock. Twenty-six months after electrode implantation, they found that early treatment prevented NDO and urinary incontinence [48]. Similar observations were made in a dog model of SCI [49]. More recently, Biardeau and co-workers demonstrated that administration of fesoterodine, a well-established antimuscarinic drug, initiated immediately after experimental spinal injury and lasting for 6 weeks, had beneficial effects on bladder function, with a significant reduction of intravesical pressures in treated SCI rats [50]. This suggest that early interventions might be used to prevent NDO and protect the urinary tract. These observations are in line with other studies, where treatment was also initiated at very early stages of neuronal injury progression, that demonstrated a positive impact on pain and spasticity [51, 52].

2.1. Effects of early bladder injection of BoNT/A in SCI rats (Unpublished observations)

BoNT/A has been in the spotlight of neurourology research for several years, and the toxin effectiveness in managing bladder hyperactivity has been proved in several research projects and numerous clinical trials [2, 3]. Bladder wall injections of Onabot/A are currently licensed for the management of NDO in SCI patients that do not respond to oral antimuscarinic drugs. Still, treatments initiate in a chronic phase post-injury, when NDO is already settled. With focus on risks of periodical increments in intravesical pressures and undesirable situations of urinary incontinence, we considered important to evaluate the toxin capacity to prevent NDO, rather than treating it during chronic stages.

We treated SCI rats with intra-detrusor Onabot/A 3 days after lesion and evaluated cystometric activity 4 weeks after. Our results suggested that when administered in a so early time-point after SCI, the toxin is unable to induce any significant improvement in bladder

function, with SCI rats developing NDO 4 weeks after lesion. This contrasts with toxin's proven efficacy in managing chronic NDO in experimental animals [53] and patients [22, 25-28]. Corroborating functional data, no alterations were found in bladder and lumbosacral spinal cord innervation as well as DRG neurons after the early treatment with the Onabot/A.

Our findings diverge from those from Temeltas and co-workers [54], demonstrating that BoNT/A improved basal pressure, frequency and amplitude of inhibited detrusor contractions in SCI rats, when administered 7 days after SCI. Accordingly, we might infer that bladder areflexia is accompanied by denervation and loss of neuronal activity, as suggested by the analysis of SNAP-25, GAP43, TRPV1 and VaChT in the rat bladder 1 week after SCI, which limits toxin internalization. Thus, a reasonable hypothesis is that 7 days after injury some regenerative mechanisms might start to occur, which guarantee a higher rate of toxin internalization and potentiate bladder effects. This remains to be explored and additional studies are needed.

2.2. Effects of early intravesical administration of RTX in SCI rats (Publication III)

Emergence of NDO is known to reflect massive neuroplastic events catalysed by spinal injury. The current paradigm assumes that during spinal shock the levels of nerve growth factor (NGF) increase in the bladder and spinal cord. This neurotrophin induces sprouting of TrkA-expressing bladder C-type sensory fibres, which greatly expand their peripheral and central processes. At the spinal cord, these afferents establish new synaptic contacts and a new micturition reflex is formed, restricted to the spinal cord and responsible for abnormal bladder function and/or DSD [55, 56].

Bladder C-type fibres are critical to NDO development and maintenance. The majority of these fibres express the TRPV1 receptor [57] and its expression is known to increase in NDO patients [58, 59]. Strong agonists of TRPV1, including RTX, have been used in the past to treat chronic NDO. These agonists are known to cause desensitization of TRPV1 [60] and, consequently, silence bladder afferents. Accordingly, intravesical administration of RTX is known

to reduce the number of incontinence episodes and increase bladder capacity in NDO patients [61-63]. Therefore, we questioned if, like sacral neuromodulation and anticholinergics, early intravesical administration of RTX would impact NDO development.

In **Publication III**, animals received intravesical RTX during spinal shock and bladder function was evaluated 4 weeks after spinal lesion and treatment. Bladder reflex activity was markedly better in RTX-treated SCI animals in comparison with animals receiving vehicle solution. Improvement of bladder function resulted from a restricted effect to the bladder, where expression of TRPV1, CGRP and GAP43 were reduced. In DRG and spinal cord, no major changes were observed. Moreover, no obvious signs of neuronal stress were found, as DRG neurons had very low levels of the stress marker ATF3 and presented normal growth capacity when in *in vitro* conditions. Thus, results presented in **Publication III** support that early administration of RTX can attenuate NDO development without any major deleterious secondary effects, as this neurotoxin only acted on the bladder. Moreover, these results also concur to a shift in the paradigm of NDO treatment, anticipating interventions rather than only acting when NDO is a fixed condition, with little chances to change.

Interestingly, our results indicate that early intervention with RTX is more effective than with BoNT/A, which failed to produce any effect on NDO development. Our observations contrast with those from a randomized study including 25 chronic NDO patients, which demonstrated better clinical and urodynamic outcomes following treatment with BoNT/A, when compared to RTX [64]. The reasons behind those contradictory results can only be speculated at present but might reside on the mechanisms of action of each neurotoxin. While BoNT/A-mediated impairment of neurotransmission requires active axonal terminals, exposing synaptic proteins upon vesicle fusion, RTX binds to TRPV1 receptors whether expressing fibres are active or silent. During spinal shock, when the bladder is arreflexic, the majority of bladder fibres are silent. Thus, following intradetrusor injection, BoNT/A fails to be internalized and is quickly degraded by extracellular matrix proteases [65]. On the other hand, although BoNT/A is

able to target a larger population of bladder fibres [32], intravesical administration of TRPV1 targets nerve fibres and TRPV1-expressing urothelial cells [36, 37] and the net-effect of RTX-induced desensitization does not depend on the activity of TRPV1-expressing structures. Therefore, pending additional studies, RTX might be preferable for early treatments aiming to harness NDO development.

Conclusions

All studies included in this dissertation aimed to improve NDO treatment and to investigate if NDO can be prevented. Work focused on two well-known neurotoxins: BoNT/A, the current gold-standard treatment, and RTX, a plant-derived neurotoxin. The main findings of these studies are:

1. The dose conversion ratio for bladder wall injection of Onabot/A and Abobot/A should be 1 U of the first for each 1.6 U of the latter.

2. Effectiveness of Onabot/A and Abobot/A treatment is dependent on variability in BoNT/A bladder wall injections, which is likely overcome by numerous injection points, and spread of the toxin across tissue.

3. Intrathecal administration of Onabot/A improves urodynamic pattern of neurogenic bladder after SCI. This route for toxin administration specifically modulates afferent sensory neurons and spares efferent remaining activity.

4. Early administration of intradetrusor BoNT/A does not affect NDO development, which was present in control and treated SCI animals.

5. Intravesical administration of RTX during spinal shock successfully attenuates NDO emergence, without secondary effects. Together with observations in point 4 and pending further investigation, it may be possible that intravesical RTX could be a better choice to harness NDO development.

References in *Final considerations*

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